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TOWARDS UNDERSTANDNING OF HUMAN CYTOMEGALOVIRUS IN GLIOBLASTOMA

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Towards understanding of Human Cytomegalovirus in Glioblastoma
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

Моей семье

Στην οικογένειά μου

Alla mia famiglia

All truths are easy to understand once they are discovered; the point is to discover them.

Galileo Galilei

ABSTRACT

Human Cytomegalovirus (HCMV) is a widely prevalent herpesvirus. Following an active primary infection, often asymptomatic or subclinical in immunocompetent individuals, the virus establishes latency. HCMV produces several hundreds of proteins with immunomodulatory and immunosuppressive abilities to interfere with the immune defenses of the host in order to stay undetected and avoid elimination. During recent decades, HCMV proteins and nucleic acids have been detected in a variety of tumors. Through a combination of various immunosuppressive abilities, HCMV has been suggested to be a pathogen potentially contributing to the development of cancer. Glioblastoma Multiforme (GBM) is the most common primary intracranial tumor in adults and is associated with poor prognosis despite treatment. It has been shown that 99% of GBM express HCMV-IE (Immediate early) epitopes and that the level of HCMV-IE expression is a prognostic factor for patient survival. Therefore the main goal of my thesis work was to characterize the immune phenotype of HCMV infected GBM patients and to investigate the possible role of HCMV in gliomagenesis. This work is important for further development of therapies against GBM.

As it is well accepted that T cells are central players in the immune response, we investigated whether HCMV subverts the immune system by directly inhibiting proliferation and activation of CD4-positive T cells (***Paper 1)***. Our observations suggest that HCMV silences T cells, which has clinical consequences for both humoral and cell-mediated immunity and may explain the general immunosuppression observed in HCMV-infected patients.

To investigate the role of HCMV in gliomagenesis, we examined the ability of HCMV to induce a more aggressive cancer stem cell (CSC)-like phenotype in primary GBM cell lines (***Paper 2)***. HCMV infection induced a stem cell phenotype in primary GBM cell lines as determined by changes in the cellular gene expression profile and by the conferred ability of cells to grow as neurospheres *in vitro*. As CSCs are known to be resistant to chemotherapy, our results imply that HCMV may enhance the malignancy grade of the tumor, and possibly contribute to therapy resistance.

To understand whether the immunological phenotype of GBM patients with HCMV infected tumors has an impact on overall survival, we investigated the T cell phenotypes of these patients (***Paper 3)***. We found that patients with lower levels of CD3-positive cells had significantly shorter overall survival. GBM patients with signs of immunosenescence, as indicated by CD57 expression and loss of CD28 expression on CD4-positive T cells, had shorter overall survival. As the CD57+CD28- CD4+T cells only have been described in HCMV seropositive individuals, our findings suggest that the signs of immunosenescence in GBM patients may be linked to HCMV infection and indicate poor patient survival.

Since HCMV is carried by a large part of the population and by most of GBM patients, we investigated the serology status, the levels of HCMV DNA and RNA in blood and T cell activity against HCMV peptides in blood of GBM patients (***Paper 4)***. All GBM patients were positive for HCMV proteins in the tumors and HCMV DNA in blood and T cell reactivity against HCMV peptides, but despite this, 29% of patients were HCMV IgG negative using three commercial ELISA tests. Some of these HCMV seronegative patients were IgG positive, when tested in an ELISA assay using antigens prepared from an HCMV clinical isolate. Thus, our results suggest that commercial serology tests are not reliable to detect an ongoing or previous HCMV infection in GBM patients.

We investigated cytokine patterns in blood of GBM patients with HCMV infected tumors and studied whether neutrophil activation is associated with HCMV infection and GBM progression (***Paper 5)***. We found that GBM patients with higher neutrophil activity had shorter time to tumor progression and shorter survival. In conclusion, considering the immunomodulatory abilities of HCMV and its CSC-phenotype inducing potential in primary GBM cell lines, our results suggest an active role of HCMV in GBM and that this virus may be capable of driving GBM progression.

LIST OF SCIENTIFIC PAPERS

I. **Fornara O*,** Odeberg J*, Khan Z, Stragliotto G, Peredo I, Butler L, Söderberg-Nauclér C
Human cytomegalovirus particles directly suppress CD4 T-lymphocyte activation and proliferation.
Immunobiology.2013Aug;218:1034-40.doi: 10.1016/j.imbio. 2013.01.002.

II. **Fornara O**, Bartek J Jr, Rahbar A, Odeberg J, Khan Z, Peredo I, Hamerlik P, Bartek J, Stragliotto G, Landázuri N, Söderberg-Nauclér C.
Cytomegalovirus infection induces a stem cell phenotype in human primary glioblastoma cells: Prognostic significance and biological impact.
Cell Death Differ. 2015 Jul 3. doi: 10.1038/cdd.2015.91.

III. **Fornara O,** Odeberg J, Wolmer Solberg N, Tammik C, Skarman P, Peredo I, Stragliotto G, Rahbar A, Söderberg-Nauclér C
Poor survival in glioblastoma patients is associated with early signs of immunosenescence in the CD4 T-cell compartment after surgery**.**
Oncoimmunology. 2015 Jun 5;4(9):e1036211.

IV. Rahbar A, Peredo I, Solberg NW, Taher C, Dzabic M, Xu X, Skarman P, **Fornara O,** Tammik C, Yaiw K, Wilhelmi V, Assinger A, Stragliotto G, Söderberg-Naucler C.
Discordant humoral and cellular immune responses to Cytomegalovirus in glioblastoma patients whose tumors are positive for CMV.
Oncoimmunology. 2015 Feb 25;4:e982391.

V. Afsar Rahbar, Madeleine Cederarv, Nina Wolmer-Solberg, Charlotte Tammik, Giuseppe Stragliotto, Inti Peredo, **Fornara O**, Xinling Xu, Mensur Dzabic, Chato Taher, Petra Skarman, Cecilia Söderberg-Nauclér
Enhanced neutrophil activity is associated with shorter time to tumor progression in glioblastoma patients
Oncoimmunology. 2016 Aug, doi: 10.1080/2162402X.2015.1075693

*Authors have contributed equally to this work

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ATRX	α-thalassemia/mental-retardation-syndrome-X-linked
ConA	Concavallin A
COX-2	Cyclooxygenase-2
DC	Dendritic cells
E	Early
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked ImmunoSorbent Assay
G-CIMP	Glioma-CpG island methylator phenotype
GBM	Glioblastoma Multiforme
GCV	Ganciclovir
GTR	Gross Total Resection
HCMV	Human Cytomegalovirus
HIV	Human Immunodeficiency Virus
HPV	Human Papiloma Virus
IDH	Isocitrate dehydrogenase
IE	Immediate Early
IHC	Immunohistochemistry
IRS	Internal repeat sequences
ISH	In situ hybridization
kbp	Kilo Base Pair
L	Late
MGMT	O6-methylguanine-DNA methyltransferase
NK	Natural Killer
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PDGFRa	Platelet-Derived Growth Factor Receptor-a
PMA	Phorbol Myristate Acetage
QNAT	Quantitative Nucleic Acid Testing
SMC	Smoth Muscle Cell
STR	Subtotal Resection
TERT	Telomerase Reverse Transcriptase
TLR	Toll Like Receptor
TMZ	Temozolamide
UL	Unique long
US	Unique short
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization

1 INTRODUCTION

1.1 GLIOBLASTOMA MULTIFORME (GBM)

GBM is the most common primary brain tumor in adults. It is classified as astrocytoma grade IV according to World Health Organization (WHO). The grading system takes in consideration many aspects of the tumor, such as cytological phenotype, mitotic activity, microvascular proliferation and others[1]. Grade IV is the most malignant tumor with nuclear atypia and high proliferation index. These tumors are necrosis-prone and associated with extensive infiltration into the surrounding tissues with rapid microvascular proliferation and fast pre- and post-operative disease evolution and fatal outcome. Grading of the brain tumors according to the WHO classification is important for choosing the proper oncological treatment[1].

1.1.1 EPIDEMIOLOGY OF GBM

GBM has a very poor prognosis with overall survival time of approximately 14.6 months despite aggressive treatments including surgery, radiotherapy with concomitant and adjuvant Temozolomide (TMZ) chemotherapy. The incidence rate of GBM is 3.19/100.000 in population per year, making it the most common malignancy in Central Nervous System (CNS) in adults. The median age at diagnosis of GBM patients is 64 years, implying high absolute numbers of cases among elderly, which, without an effective treatment, will keep increasing with growing and aging populations. GBM is uncommon in children, representing only 3% of all CNS and brain tumors among 0 to 19 year old patients [2]. Primary GBM is more common in men compared to women with the ratio 1 vs 0.33 and it is twice as common in Caucasians compared to Afro-Americans [3]. The highest relative survival is one year post diagnosis and is 36.7% in men and 32.8% in women. In the second year it declines to 13.7% in both genders and continues to decline to less than 5% at 5 years post diagnosis. The peak of incidence of mortality among GBM patients occurs in the first quarter of the second year post diagnosis [3, 4]. Numerous environmental factors have been investigated, but no direct risk factor has been identified and GBM is therefore considered to be sporadic[3]. Allergies and atopic diseases have been associated with lower GBM risk as well as short-term (<10 years) usage of anti-inflammatory medication [5, 6]. Inconsistent and inconclusive results have been recently published regarding the link between mobile phone usage and risk of glioma[7, 8]. Competing comorbidities and poor performance status at baseline predict not only shortened survival but also limited treatment tolerance[9]. In general, many factors affect the prognosis of patients with GBM, among them are age, other comorbidities of patients, preoperative performance status (both cognitively and physically), tumor location, size and extent of surgical resection[10, 11].

1.1.2 THE ORIGIN AND TYPES OF GBM

GBM is a richly vascularized, highly invasive and infiltrating tumor. GBM tumors exhibit robust proliferation, high grade of angiogenesis, genetic instability, and immunosuppression in GBM patients. It is rarely cured and very prone to reoccurrence[12].

Major GBM locations are supratentorial regions: frontal, temporal, parietal, and occipital lobes. GBMs are rarely seen in the cerebellum and are very rare in the spinal cord[2, 13, 14]. Patients with frontal lobe GBMs have longer survival time compared to patients with GBMs in other supratentorial locations[15, 16]. Distal metastases of GBM are extremely rare and have only been reported in 0.44% of the cases[17]. Most probably, the low evidence for extracranial metastasis of GBM is partially due to the short lifespan of GBM patients but also because of the fact that GBM cell escape is restricted by the limited lymphatic transport in the brain[17]. Recent studies in mice have demonstrated an existence of meningeal lymphatic vessels in the CNS that drain cerebrospinal fluid and contribute to immunity in the brain[18]. However, these findings are still to be confirmed in humans.

Based on different genetic pathways, GBM phenotypes can be divided into primary *(de novo)* and secondary subtypes, affecting patients in different ages and having different initial treatments. Primary GBM occurs mainly in older patients (mean age, 62 years) and account for 80% of all GBMs. Younger patients (mean age, 45 years) suffer mainly from secondary GBMs, which develop from astrocytmas or oligodendrogliomas[2]. Recently, another GBM type has been added by the WHO "GBM with oligodendroglioma component". This GBM type has a cytological phenotype with areas resembling anaplastic oligodendroglioma, but with the typical characteristics of GBM such as necrosis with or without microvascular proliferation[1].

Recent profiling has divided GBMs into different molecular phenotypes: proneural, neural, classical, and mesenchymal, according to genetic alteration in EGFR, NF1, and PDGFRA/IDH1 genes. Each of these subtypes shows an enrichment of gene expression signatures from distinct neural lineages, implying that the expression patterns of the different subtypes may reflect the phenotype of their specific cells of origin[19].

1.1.3 MOLECULAR MARKERS OF GBM

MGMT promoter methylation status

Temozolamide (TMZ) is an alkylating agent that causes DNA damage and cell death. It is used as standard chemotherapy treatment for GBM patients. O6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair protein, which removes alkyl groups from DNA and makes GBM cells resistant to TMZ. Methylation of MGMT promoter leads to silencing of repair functions and increased sensitivity for TMZ[20]. Methylation status of the MGMT promoter is an important marker for chemotherapy resistance. The MGMT promoter is methylated in approximately 50% of newly diagnosed GBMs[21, 22]. Higher levels of MGMT methylation have prognostic significance and patients with higher methylation levels

of MGMT promoter have higher overall and progression-free survival as well as better response to alkylating chemotherapies[23-25]. Recent study demonstrated that activation of Wnt/β-catenin pathway has a potential to activate MGMT expression in mice. Thus inhibition of Wnt pathways leads to inhibition of MGMT expression and increases cancer sensitivity to DNA-alkylating drugs[26].

IDH mutation

Isocitrate dehydrogenase (IDH) is an enzyme, which catalyzes the decarboxylation of isocitrate to α-ketoglutarate. There are three known isoforms of IDHs: 1, 2, and 3[27]. IDH mutations are generally more common in astrocytomas and oligodendrogliomas but IDH1 mutations were observed in higher frequencies in GBM patients [28-30]. IDH1 mutations induce an extensive DNA hypermethylation in GBM. IHD1 mutation is typical for secondary GBMs and is often used as a marker of distinction of secondary GBMs. Secondary GBMs with IDH1 mutation are believed to have developed from lower grade astrocytomas and oligodendrogliomas and have a distinctive phenotype with frontal location, less radiographic contrast enhancement and less necrosis. Patients with IDH1 mutated GBMs have better prognosis compared to those without IDH1 mutation[31].

Glioma-CpG island methylator phenotype (G-CIMP) is a DNA methylation phenotype present in approximately 10% of GBMs and is associated with IDH1 mutation in GBMs of proneural subtype. Patients with IDH1 mutation and G-CIMP phenotype show longer overall survival[32, 33].

EGFR amplification

Epidermal Growth Factor Receptor (EGFR) is a transmembrane tyrosine kinase, which when activated modulates various cellular pathways including survival, migration and proliferation[33]. In GBM, EGFR activation induces tumor growth, invasiveness and therapy resistance[34, 35]. EGFR activity can be induced by various means, such as genetic mutations or alterations of EGFR gene, EGFR amplification, inhibition or deletion of downstream pathway inhibitors and other factors [36, 37]. About 40% of GBMs have EGFR amplification and it seems to be more common in primary GBMs. Unfortunately, there is no consensus about EGFR amplification in the context of patient survival, since clinical studies showed no direct correlation to the overall survival rates[38-40] [41]. This can be explained by the fact that GBMs with EGFR amplification are a heterogeneous group of tumors and survival might be affected by the degree of amplification. However, since EGFR activation in GBM may contribute to invasiveness and tumor growth, it further contributes to tumor progression and poor survival of GBM patients.

p53 mutation

The tumor suppressor p53 protein is a nuclear transcription factor, which modulates transcription of different genes important for cell differentiation, apoptosis and cellular responses to DNA damage and senescence. Murine Double Minute 2 (MDM2) protein has

been shown to block p53 activity by directly binding to p53 and forming a complex, which leads to inhibition of p53 transactivation[42]. p53 is activated during cellular stress and helps in DNA repair and other cellular reparation processes. All mutations in the p53 gene lead to inhibition of repair activity of this protein and may further lead to uncontrolled cell proliferation and tumor progression[43]. Mutations of the p53 gene are more prevalent in secondary GBMs (60-70%), than in primary (25-30%) GBMs[44].

Germ line mutations of the p53 gene are typical for Li- Fraumeni Syndrome in which patients are predisposed to multiple neoplasms including brain tumors[45]. This indicates that the loss of p53 function can contribute to the malignant transformation and development of cancer.

ATRX mutation

α-thalassemia/mental-retardation-syndrome-X-linked (ATRX) is a chromatin-remodeling factor. Mutations in the ATRX gene have been observed in different tumors[46], but are present mainly in tumors with astrocytic origin and are clustered with IDH 1/2 and p53 mutations[47]. ATRX mutations may cause alternative lengthening of telomeres, through telomerase-independent pathway for telomere maintenance, which leads to genetic instability and tumor progression [48].

TERT mutations

The TERT gene encodes for telomerase reverse transcriptase (TERT), which is important for telomerase activity and therefore essential in cell proliferation and apoptosis[49]. TERT promoter mutations have been reported in different tumors such as melanomas, pancreatic carcinoma and others, but are common mutations in GBM and are most frequently seen in primary GBMs[50]. In GBM, TERT mutations are significantly correlated with EGFR amplification but have an inverse correlation with IDH and p53 mutations[49, 50]. Patients with TERT mutations in their GBMs tend to have shorter survival than those without TERT mutations, however when adjusted for the primary or secondary subtypes, there is no significant differences in overall survival[50].

Genetic losses of chromosomes

In 80-90% of GBMs a part or the whole chromosome 10 is lost[44]. However, in many studies the deletion in the long arm of chromosome 10 (10q) has shown controversial results when correlated to patient survival[51-54]. A tumor suppressor gene called phosphatase and tensin (PTEN) gene is located on chromosome 10 and is either mutated (in approximately 20-40% of GBMs) or lost due to chromosomal changes in GBM [53].

1.1.4 TREATMENT POSSIBILITIES OF GBM

During the last decade, advances have been made in the treatment of GBM. There is a lot of ongoing research aimed to improve the dismal overall survival and the quality of life of GBM patients. Current treatment strategies and novel approaches are summarized in figure 1 and discussed further below in detail.

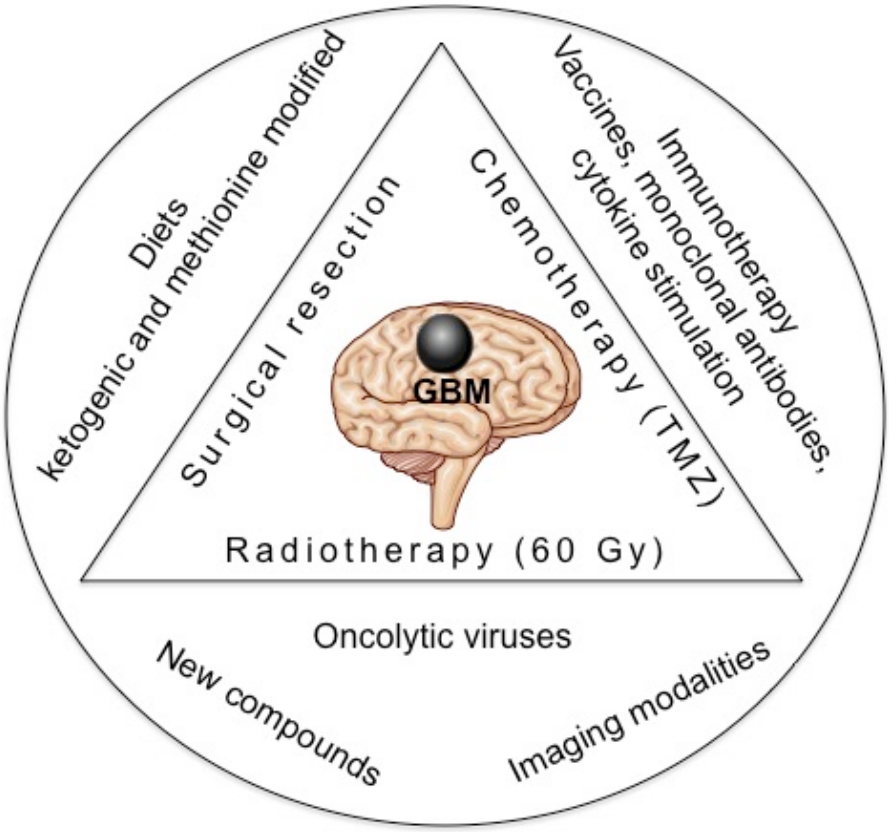


Figure 1. Summary of treatment possibilities for GBM. *The standard treatment consists of chemotherapy, radiotherapy and surgical resection. Many new strategies are under development for treatment of GBM. For example: oncolytic viruses, immunotherapy, vaccines and others.*

Standard treatment

Despite intensive multimodal treatments, GBM becomes therapy resistant, progresses and patients have an overall survival of only approximately 14,6 months [2]. Current standard therapy of GBM consists of initial surgical resection, followed by fractioned radiotherapy (a total of 60Gy divided into 30 fractions, 2Gy/fraction, during 6 weeks) with concomitant and adjuvant chemotherapy mainly with TMZ [55]. Surgical resection of GBM is classified as gross total resection (GTR) and subtotal resection (STR) when complete removal of the tumor is not possible. The standard treatment is based on the study conducted by the European Organization for Research and Treatment of Cancer and National Cancer Institute of Canada Clinical Trials Group (EORTC and NCIC) including 573 patients in 85 centers worldwide. This study showed significantly increased 2 and 5-year survival rates in groups that combined combined radio- and chemotherapies after the surgery. Additionally, patients with MGMT promoter methylation had better results of the combined treatment and higher 5-year survival rates[56, 57].

There is increasing evidence that a complete resection of GBM with decompressive and cytoreductive effects has significant survival advantages [58]. Patients with tumor resection of more than 90% have significantly higher one-year survival compared to those with less than 90% tumor resection [59]. It is therefore of highest importance to optimize the surgical

removal of the entire macroscopic tumor. For optimization of surgical resection 5-aminolevulinic acid (ALA) is used in clinical settings. ALA stimulates synthesis and accumulation of fluorescent porphyrins in malignant tissues. Tumor fluorescence derived with the help of ALA enables more complete resections of contrast-enhanced tumors, which leads to improved progression-free survival in patients with malignant gliomas[60]. 65% of surgical resections using ALA have led to GTR, while only 36% reached GTR criteria using conventional surgical methods [61]. However, it is well known that radical surgical resection of GBM is challenging due to infiltrating growth of the tumor into the surrounding normal brain tissue. Total tumor removal is often impossible without postoperative functional and neurological impairments. It depends on the size and the location of the tumor as well as the vicinity to important functional centers in the brain. Unfortunately 80-90% of GBM recurrences after surgery, chemotherapy and radiotherapy occur within the original treatment field of the primary tumor[62-66]. This implies that the main cause of treatment failure is the inefficacy to control the tumor at the original site. Consequently novel therapies are needed to combat the tumor growth at the primary site.

Novel therapies

For a long time, CNS in general and the brain in particular were thought to be immune-privileged. However, during recent years novel immunotherapies are being investigated as a possible targeted therapy for GBM. These include passive immunotherapies using monoclonal antibodies against tumor associated antigens, cytokine stimulation with IL-12[67] and active immunotherapies such as peptide vaccines[68] and DC-based vaccines[69]. The blood brain barrier remains the major limiting factor in the development of immunotherapies for GBM treatment. The monoclonal antibody Avastin (bevacizumab) that is currently used in clinical settings is an antibody against vascular endothelial growth factor (VEGF). During rapid growth GBMs secrete VEGF to promote neoangiogenesis and Avastin blocks the response to VEGF, hereby preventing neovascularization of the tumor and inhibiting tumor cell proliferation[70]. Because of the systemic blockage of EGFR one of the uncommon but feared side effects of Avastin is deep vein thrombosis[71]. In recent studies, Avastin unfortunately failed to show improved overall survival in patients treated with Avastin as an-add on to standard therapy[72]. Another promising therapy is the peptide vaccine Rindopepimut that targets mutated EGFR, present in approximately 30% of GBMs. Rindopepimut induces a strong immune response, which aims to eliminate cells with EGFR mutations. Rindopepimut has a low toxicity profile, is well tolerated by patients and has few side effects. When Rindopepimut was added to standard GBM therapy it showed impressive survival improvements with a median progression-free survival of 9.2 months and a median overall survival of 21.8 months from study entry (approximately 3 months after diagnosis)[73].

During the last decade, the field of virology has also offered some advances for GBM treatment. Oncolytic viruses are normally replication-incompetent except when present in cancer cells. Oncolytic viruses recognize the tumor cells through certain receptors such as

EGFRvIII, PDGFR, and IL-13R and after viral entrance into the cancer cells undergo lytic expansion and destroy the tumor cell population[74]. To date modified adenovirus have been investigated in clinical trials and showed promising results. Preliminary results demonstrate that intratumoral injection of adenovirus DNX-2401 resulted in either stable, partial, or complete regression of tumor in 52% of 24 GBM patients included in the trial [75].

Imaging modalities for GBMs are also undergoing tremendous advances, which will increase the possibility for better characterization and understanding of tumors before surgery[55].

Even different diets have been proposed as a complement to standard therapy for GBM. For example, the ketogenic diet has been proposed as a supportive metabolic therapy. Ketogenic diet is a high-fat and low-carbohydrate diet providing ketone bodies instead of glucose, which is unfavorable for the tumor cells[76]. Another proposed diet is methionine-modified diet due to dependence of cancer cells on exogenous essential amino acids such as methionine [77]. Unfortunately none of these diets so far showed improved survival in GBM patients when combined with standard treatments[74].

1.1.5 IMMUNOSUPRESSION IN GBM

GBM patients have an altered immune phenotype and are often considered to be immunosuppressed. GBM tumors have multiple mechanisms for suppression of antitumor immune responses as summarized in Figure 2 and discussed further below.

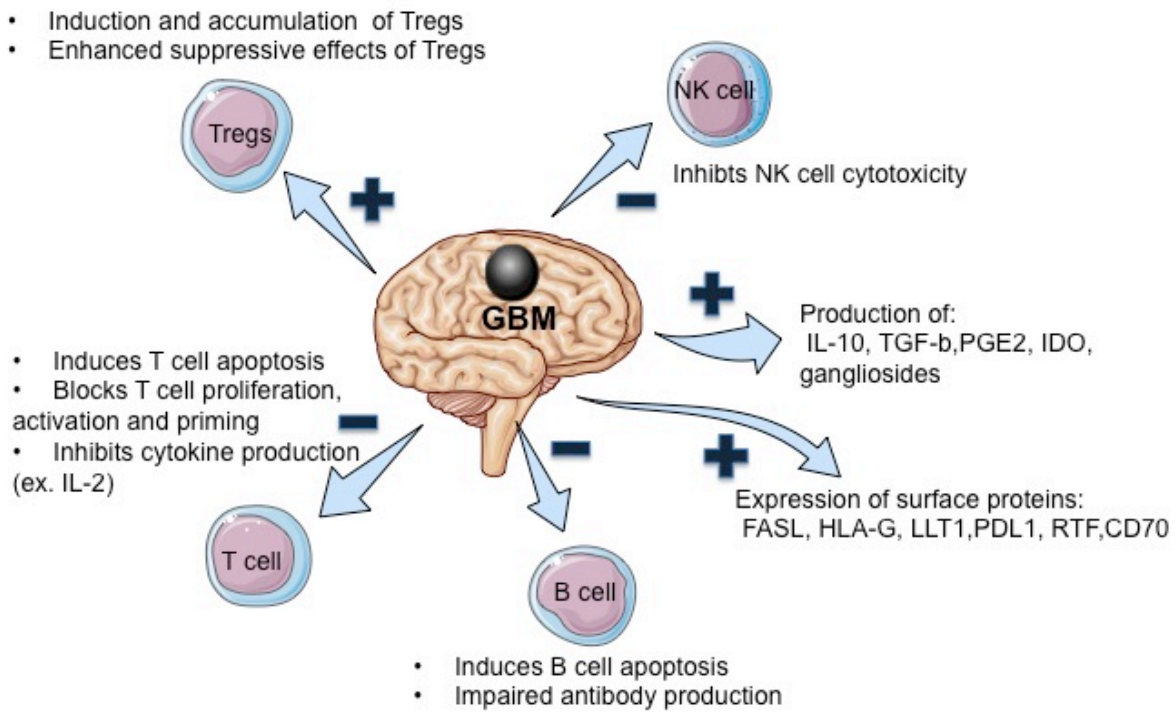


Figure 2. Summary of immunomodulatory abilities of GBM. *GBM affects different immunological cell types such as regulatory T cells (Tregs), Natural killer cells (NK cell), T and B cells and induces expression of some surface proteins, which modulate different cell functions.*

Immunosuppressive cytokines in GBM

GBM produces multiple immunosuppressive cytokines in order to interfere with the antitumor responses in GBM patients. It has been proposed that TGF-β, IL-10 and prostaglandin E2 (PGE-2) are important contributors to immune suppression in GBM patients as well as in patients with other tumors. These cytokines have multiple immunosuppressive abilities such as inhibition of antitumor effector cell responses as well as inhibition of T, B and NK cell functions and generation of Tregs [78]. These cytokines-induce an immunosuppressive environment in GBM and may contribute to reduced B cell counts and impaired B cell antibody production[79, 80]. In addition, TGF-β and PGE-2 may be involved in tumor proliferation, while PGE-2 itself may promote angiogenesis and tumor cell migration[81]. It has been shown that regions of GBM tumors with high extracellular TGF-β concentration have fewer numbers of infiltrating lymphocytes than regions with lower levels of TGF- β [80].

Tumor associated macrophages (TAMs) in GBM

Increasing evidence suggests that TAMs play an important role in balancing between inflammatory and immunosuppressive responses through different phenotypes of macrophages (M1 and M2). IL-10 and TGF-β may induce TAMs to acquire M2 properties thus stimulating immune suppression and tumor proliferation[82]. In fact, GBM tumors with more aggressive phenotypes showed increased M2 infiltration[83].

T cell responses in GBM

It has been observed that GBM patients suffer from lymphopenia of CD4 T cells with the CD4/CD8 ratio closer to 1. They also suffer from a decreased production of and hyporesponsiveness to interleukin 2 (IL-2) by T cells. GBM patients have elevated levels of Tregs, which are thought to be recruited to the tumor location by CCL2/22 chemokines secreted by GBM cells[79, 84-86]. These Tregs may inhibit T-cell proliferation and activation. Tregs are also believed to secrete immunosuppressive cytokines (TGF-β, IL-10) and are able to further differentiate and perform a perforin/granzyme- mediated killing of responder T cells, or modulate antigen presenting cell function [87]. Tregs also express the endonucleases CD39 and CD73, responsible for extracellular synthesis of adenosine which also has immunosuppressive properties[88]. Indoleamine 2,3-dioxygenas IDO is an intracellular tryptophan catabolizing enzyme and is overexpressed in GBM. It is involved in inhibition of T cell proliferation, especially in Tregs[89].

The role of microglia in GBM

Microglia cells are abundantly present in GBM tumors because GBMs produce chemoattractants for microglial cells. Microglia in GBM are also sources of metal proteases and growth factors such as EGF and VEGF, which are essential for angiogenesis, proliferation and local spread of GBMs. Microglia play an important role in local immune

suppression in GBM by production of IL-10 and expression of apoptotic inducers such as ligand-1 and FAS ligand (CD178)[90].

Immunosuppressive surface molecules in GBM

GBM cells express multiple surface molecules that may directly inhibit immune effector cells. For example, the expression of CD70 on the GBM cells induces apoptosis of CD27-expressing T and B cells[91]. The expression of FAS ligand (CD178) on the GBM cells induces apoptosis of FAS (CD95)-expressing lymphocytes[92], which results in effective escape from the cellular immune response. Another surface molecule, which is overexpressed in GBM is PDL1 (CD278). PDL1 inhibits T cell functions, such as proliferation, cytokine production and cytotoxicity and may induce T cell apoptosis[89]. Lectin-like transcript 1 (LLT1) is also overexpressed on the surface of GBM and inhibits CD161-induced NK cell cytotoxicity. Interestingly, TGF-β may induce LLT1 expression in GBM [93]. Regeneration and tolerance factor (RTF), which was first described as a surface protein of cytotrophoblasts of early placentas involved in T and NK cell function is also overexpressed by GBM cells, inhibiting NK and T-cell mediated cytotoxicity [94]. GBM cells downregulate MHC class I and show high surface expression of the nonclassic MHC class 1b molecule, which leads to inhibition of proliferation and cytotoxicity of T cells and NK cells[95].

All these immunosuppressive mechanisms observed in GBM patients may contribute to an impaired ability to combat cancer and lead to tumor progression and poor survival.

1.1.6 STEM CELLS IN GBM

After surgical removal and additional radiochemotherapy, GBMs have an ability to return. This clearly portraits the aggressive nature of the GBM tumor and highlights the possible existence of cancer stem-like cells, which posses self-renewal and multipotent differentiation abilities and may contribute to therapy resistance. These cells have been identified in GBM and are called Glioma Stem Cells (GSC)[96-98]. Because of pluripotency, GSCs are able to differentiate in various cell lineages and contribute to heterogeneity and complexity of the GBM tumor. It has been suggested that GSCs are spared by radio- and chemotherapy because of their ability to remain quiescent and they are believed to induce tumor recurrences[99]. It has been shown that GSCs in GBMs in part resemble normal neural stem cells[100]. GSC populations are lacking the expression of neural differentiation markers, which indicates mutagenic transformation from neural stem cells to cells with multiple differentiation abilities that resemble malignant cells [96].

GSC surface markers in GBM tumors

One of the most commonly suggested markers of Glioma stem cells is Prominin 1 (also called CD133), which is a surface glycoprotein that is expressed on both normal neural stem cells and GSCs. Although the function of CD133 is poorly understood, the silencing of CD133 expression in GBM cells blocks the carcinogenic and self-renewing capacity of neurosphere-forming GSC[101]. It has been observed that especially neural and

mesenchymal GBM subtypes have increased expression of CD133[102]. Mesenchymal GBMs are most aggressive types of GBM and are highly resistant to radiotherapy [103]. The possible reason to this enhanced aggressive phenotype may be the fact hat mesenchymal GBMs have higher levels of CD133+ GSCs. However it has been shown that even CD133- cells could generate tumors to some extent [104]. CD133+ GSCs grow non-adherently in spheres under conditions that permit stem cell proliferation. Each sphere is thought to originate from one single cancer stem cell that produces daughter cells with phenotypes of GBM[105]. GSCs have an ability of asymmetrical division and thereby give rise to more differentiated daughter cells with higher proliferative capacities, contributing to the heterogeneity of the tumor and to tumor recurrence after radio- and chemotherapy[100].

Activation of intracellular signaling pathways in GSC

GSCs are known to be the major contributors to radio- and chemotherapy resistance of GBMs. In glioma xenografts subjected to radiation, there is an increase in CD133+ cells, suggesting that radiotherapy selects the GSCs population. GSCs are believed to activate several DNA damage checkpoint proteins such as ATM, Rad17, Chk2, and Chk1 in response to radiation. This ability of GSC contributes to efficient DNA repair and better post radiation recovery of GSCs. [106]. Another important pathway for radiation resistance is controlled by Notch signaling. Notch signaling promotes self-renewal in GSCs and suppression of the Notch pathway increases sensitivity of GSCs to radiation, which suggests a clinical significance of this pathway [107]. Another molecule overexpressed in GBMs is signal transducer and activator transcription 3 (STAT3). STAT3 is involved in various cellular processes, such as cell growth, division, and apoptosis, both in normal stem cells and in GSCs. Abnormal expression of STAT3 in GSCs promotes cell growth and contributes to immunosuppression [108]. A typical genetic alteration in GSC is the knockdown of microRNA (miR-145) expression that normally functions as a tumor suppressor. The knockdown of miR-145 induces cell proliferation, invasion and migration The inhibition of miR-145 expression in GBMs is associated with poor patient outcome [109]. It has been proposed that GSCs use the same transcription factors as the normal stem cells, such as sex determining region Y-Box (SOX2), octamer-binding transcription factor 4 (OCT4), and Nanog homeobox (NANOG). Theses transcription factors are critical components in maintaining pluripotency, self-renewal and proliferative capacity and repressing cell differentiation both in normal stem cells and in GSCs[110, 111]. The silencing of SOX2 in human GBM cells transplanted in immunodeficient mice ceased cell proliferation and resulted in loss of tumorigenicity [111].

Tumor vascularization and GSC

GBMs are highly vascularized and it has been speculated that GSCs are mostly present in the vascular niche of the tumor, where endothelial cells may promote the self-renewal and undifferentiated state of GSCs. Some studies suggest that an increase of the vasculature or the number of endothelial cells in orthotopic brain tumor xenografts, increased GSC populations. On the other hand, when anti-angiogenic therapies blocked vessel formation in the brain

tumor xenographts, formation of GSC population and tumor growth were inhibited[112]. Vascular endothelial cells also release nitric oxide (NO). NO has been shown to promote self-renewing capacity and maintain GSCs through activation of the Notch signaling pathway, which contributes to GBM therapy resistance [113].

The typical characteristics of GSC with its multipotency, quiescence and self-renewal ability contribute to the aggressiveness, tumor progression and recurrence of GBM. It is therefore of highest importance to target not only highly proliferative GBM cells but also GSCs for achievement of long-lasting remission or even cure of GBM.

1.1.7 IS THERE A ROLE FOR HCMV IN GBM?

During the recent decade there has been a lot of discussion about Human Cytomegalovirus (HCMV) infection in GBM. It was first described by Cobbs et.al in 2002 and thereafter many scientific groups have reported HCMV infection in GBM with detection of viral nucleic acids and proteins in these tumors but not in their surrounding tissues[114-117]. The level of HCMV infection in GBM has been associated with poor survival, implying that HCMV may have a direct role in the pathogenesis of this disease[118]. Despite evidence of the presence of HCMV in various tumors, including GBM, this topic is highly debated [119]. The main reason for this is the fact that several groups have failed to detect HCMV in GBM. However, the undeniable evidence of HCMV in GBM is proved by the fact that HCMV-specific T cells directed against pp65protein can recognize and kill autologous GBM cells[120]. Additionally, cellular immunotherapy with autologous T-cells directed against HCMV in GBM significantly increased recurrence-free survival[121]. Another evidence is that stimulation of dendritic cells with GBM tumor lysates leads to expansion of HCMV-specific T cells in GBM patients, indicating the presence of HCMV peptide epitopes in GBM tumors[122]. In addition, one of the articles included in this thesis reveals that 29% of GBM patients have higher levels of HCMV activity, indicated by IgM levels in their blood, compared to none in healthy controls. Taken together these observations suggest that HCMV is most probably present in GBMs and even other tumors. Groups that failed to detect HCMV in tumors have not used optimal protocols for virus detection.

HCMV's presence in GBMs and in other tumors raise many questions: what role does the virus play in the pathogenesis of cancer and, considering the ability of HCMV to interfere with cellular processes, can it contribute to progression, spread, therapy resistance and recurrence of GBM and other tumors? Furthermore, this virus affects all the arms of the immune system and causes immunosuppression. What role does this virus play in the immunosuppression GBM patients suffer from? More knowledge about the virus and its capabilities to affect cell and immunology functions is needed to understand the role of HCMV in the pathogenesis of GBM and for developing novel therapies. As HCMV is highly immunogenic, it is of utmost importance to evaluate the possibility of using the presence of HCMV in GBM in the design of immunotherapy protocols, targeting the epitopes of the virus and thereby killing cancer cells.

Remarkably, when antiviral therapy is used for the treatment of HCMV-infected tumors both in pre-clinical and clinical studies, it results in suppressed tumor growth and improved patient survival. Anti-HCMV treatment in animal models reduces neuroblastoma growth[123] and medulloblastoma growth[124] and significantly reduces GBM growth[125]. It has been demonstrated by our group that treatment of GBM patients with anti-HCMV drug as an add-on to standard therapy significantly increased patient survival. The study demonstrated that the 2 year survival was 70% among patients receiving at least 6 months of anti-viral therapy with Valganciclovir and 90% among patients with continuous treatment compared to 18% in contemporary controls. The median overall survival was 56.4 months in Valganciclovir treated patients compared with 13.5 months in the control group[126]. These observations suggest a role of HCMV in GBM and that targeting the virus in GBM tumors may open for new treatment possibilities for GBM patients.

1.2 HUMAN CYTOMEGALOVIRUS (HCMV)

HCMV is a herpes virus belonging to the Betaherpesviridae subfamily. Herpes viruses are prevalent in most vertebrates. 200 types have been identified and 8 of them are known to cause disease in humans: herpes simplex viruses 1 and 2, varicella-zoster virus, EBV (Epstein-Barr virus), human cytomegalovirus, human herpes virus 6, human herpes virus 7, and Kaposi's sarcoma-associated herpes virus [127]. Herpes viruses are well adapted, replicated only in certain types of cells and have numerous mechanisms for surviving the detection by the host immune system. The capabilities of HCMV in changing the cellular processes may contribute to various pathologies, such as chronic inflammatory diseases, autoimmune diseases and cancer.

1.2.1 STRUCTURE OF HCMV

HCMV is 200-300 nm in diameter and has a typical herpes virus structure. It consists of double stranded DNA packed in an icosahedral nucleocapsid that is surrounded by proteinaceous tegument and an outer lipid bilayer membrane, as schematically shown in Figure 3 [128].

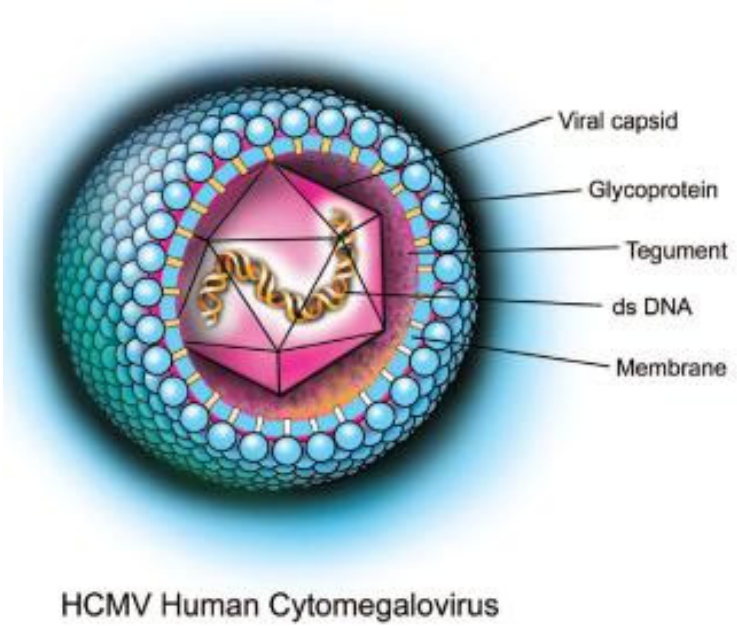


Figure 3. The structure of the HCMV virion. *Image taken from[129]*

HCMV is species-restricted and therefore it has adapted in order to survive in humans. The genome of HCMV consists of 235 kbp containing 252 Open Reading Frames (ORF) and encodes for more than 750 proteins [130]. Internal repeat sequences divide the genome of HCMV into segments named the unique long (UL) and unique short (US) regions, hence the name of the genes and proteins, depending on their location in the genome [131]. Interestingly, just 50 viral proteins are needed for further viral replication, implying that the vast majority of the proteins are produced for sustaining the virus in the host environment by interfering with the immunological defenses of the host for undetectable coexistence [132].

The viral capsid consists of five different proteins. During viral replication, three different capsids can be formed, named A, B and C capsids. A capsids consists only of a capsid shell, B capsids includes capsid shell with assembly proteins and C capsids are a complete capsids with the shell containing the DNA genome. Mature virions are formed only with C capsids containing virus DNA while A and B capsids form non-infectious virus particles and dense bodies[133]. Dense bodies formed during defective encapsulation are considered to be very immunogenic and have been proposed as anti-HCMV vaccine targets[134].

A thick protein layer, the tegument, surrounds nuclear capsids before they become enveloped. The tegument contains more than 30 proteins, of which most are phosphorylated. Tegument proteins contain more than half of the protein mass of the virion [135]. The most abundant HCMV tegument proteins are pp65, pp71, pp28 and pp150. Pp65 is the most abundant tegument protein and can confer early immune evasion mechanisms during HCMV infection[136]. Pp71 is important during the initiation of virus replication through activation of immediate early gene expression at the beginning of the lytic infection [137]. Both pp150 and pp28 are highly immunogenic proteins and are essential for assembly and egress of virus particles. Pp150 is also essential for incorporation of nucleocapsids into virus particles. It stabilizes the capsids and directs their movement in the cytoplasm during lytic infection[135]. Pp28 is essential for the cytoplasmic envelopment of tegument proteins during lytic infection[138].

The outer envelope is a lipid bilayer derived by virus budding from hosts endoplasmic reticulum-Golgi compartment and contains more than 20 different viral glycoproteins together with glycoproteins of the host cell. These include glycoproteins gB, gH, gL, gM, gN, and gO. The glycoproteins of the envelope play an essential role in viral maturation, entry into the host cell and cell-to-cell spread [139]. This mechanism of envelope assembly ensures incorporation of the host's glycoproteins in the viral envelope, thus contributing to impaired immunological recognition of the infected cells.

1.2.2 REPLICATION AND LATENCY OF HCMV

Replication cycle of HCMV

HCMV has the ability to infect many different cell types such as endothelial cells, epithelial cells, smooth muscle cells (SMC), placental trophoblasts, neurons and glial cells in the developing nervous system[140]. All herpes viruses have well preserved mechanisms of entry into the cell, as all of them express gB and gH/gL glycoproteins on the surface. These glycoproteins are essential for virus' attachment and cell entry[141]. HCMV enters the cells either through endocytosis or direct fusion with the cell membrane, as illustrated in Figure 4.

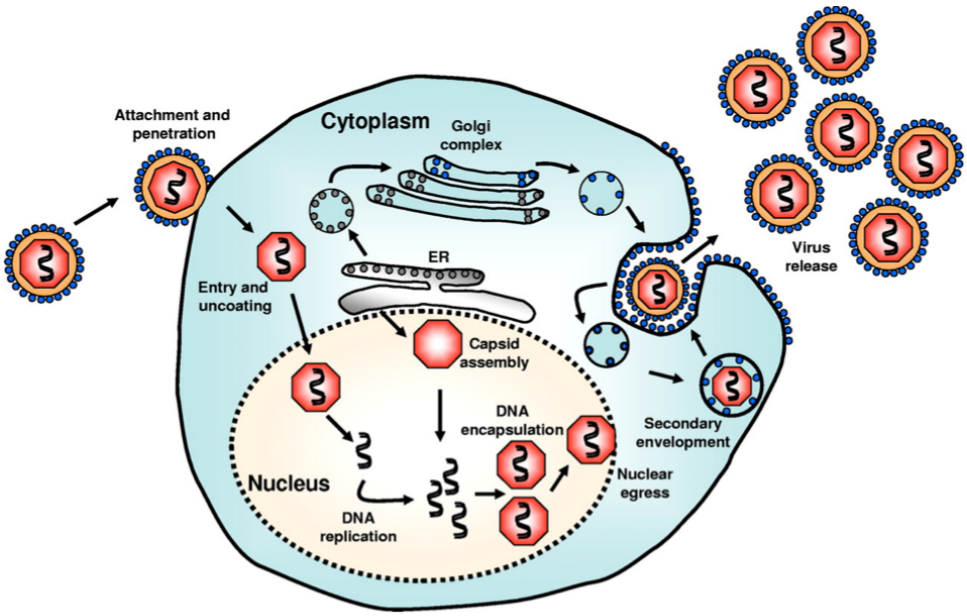


Figure 4. Lytic cycle of HCMV. Image modified from [139]

Various cell receptors have been proposed to be involved in the process of cellular entry of HCMV, such as aminopeptidase (CD13), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor-a (PDGFRa) and integrins (α2β1, α6β1 and αvβ3) [142, 143]. However none of these receptors have been found to be absolutely necessary for entry in all the infected cell types[144].

Viral attachment leads to fusion of the envelope with the cellular membrane and release of nucleocapsids into the cytoplasm. The nucleocapsids then travel through the cytoplasm and are translocated to the nucleus, followed by viral DNA release into the nucleus and highly organized viral protein expression [145]. Immediate early (IE) genes are expressed first, followed by early (E) and late (L) genes[146]. IE proteins are produced during the first hours of HCMV infection, while a complete viral replication requires 48-72 hours. The most abundant IE proteins are IE1 and IE2 and they are essential for subsequent viral gene expression and efficiency of viral replication. IE proteins regulate subsequent gene expression of the HCMV genome, acting as transactivators or autoregulators [147]. Early genes encode proteins essential for viral replication, while delayed early and late genes encode mainly structural proteins [148].

When HCMV completes the replication cycle in the nucleus, the viral DNA is packed into the newly synthesized capsids and transported from the nucleus to the cytoplasm. While travelling through the cellular compartments it acquires the tegument proteins and the lipid envelope. Secondary envelopment occurs in the cytoplasm at in the endoplasmic reticulum-Golgi intermediate compartment[139].

Latency of HCMV

All herpes viruses have an ability to establish life long latency in the host after primary infection. After an active infection, HCMV establishes latency in the CD34+ haematopoietic cell population in the bone marrow and in cells of the myeloid lineage, such as granulocyte-macrophage progenitor cells and peripheral monocytes[149, 150]. In the past, latency was considered a passive process with very little or almost non-existent viral protein production, but recent studies showed that latency is a highly active process. HCMV has different gene and protein profiles during latent and lytic infection. It has been shown that there is a small set of genes that is transcribed both during lytic infection and latency. One of these genes is LUNA and its transcription is directly dependent on the HCMV-IE proteins [151]. Additionally, virus-encoded proteins LUNA and pUL138 produced during HCMV latency may trigger activation of CD4+ T cells and production of IL-10 and IFN-γ. IFN-γ may contribute to promotion of macrophage differentiation and thereby HCMV replication and reactivation[152]. Furthermore, UL144 protein is also expressed in latently HCMV-infected monocytes but its expression is strictly dependent on the presence of a functional transcription factor GATA-2. In the same study it was demonstrated that the LUNA gene is also regulated by GATA-2 [153], suggesting a key role of this transcription factor in latency-associated protein expression. Other examples of latency associated proteins are: US28 and ORF94, which are needed for manipulation of the cellular environment in order to redirect the immune response, UL44 and UL138 block cellular pathways to immune recognition[154]. HCMV also produces latency-associated cmvIL-10, which is a homologue of the immune inhibitory cytokine IL-10 that helps the virus avoid recognition by the immune system and clearance during latency[155]. Recent studies suggest that HCMV has non-coding RNAs that are transcribed during latency [156]. Expression of non-coding RNAs during latency provides advanced mechanisms for modulating the host cell environment without attracting an immune response against the latently infected cell. During latency, HCMV establishes a certain transcriptional profile in the latently infected cells and this profile seems to be rather heterogenic, depending on the cell type[156, 157]. Therefore, the latent gene expression is most probably dictated by the cellular transcriptional milieu, implying different HCMV genetic profiles in different cell populations.

Latent HCMV can be reactivated by inflammation and stress. A key event is cellular differentiation, when monocytes differentiate into macrophages or dendritic cells (DC), upon stimulation by certain cytokines, such as TNF α, IFNγ and GM-CSF[158]. Not all cells permit active infection after virus entry. The differentiation grade of the host cell seems to play a role in viral replication. Certain differentiated cells permit viral replication while some undifferentiated cells are non-permissive for HCMV replication[159]. For example, macrophages permit an active HCMV infection, while monocytes do not. Thus the virus is reactivated when monocytes differentiate into macrophages.

1.2.3 EPIDEMIOLOGY AND TRANSMISSION OF HCMV

HCMV represents a very common infection worldwide, exhibiting a seroprevalence of 40–100% depending on geographical location and socioeconomic status. Primary HCMV infection in healthy individuals is usually asymptomatic or with mononucleosis-like symptoms, resulting in the establishment of a lifelong latency. In immunocompromized individuals, such as acquired immunodeficiency syndrome (AIDS) patients, stem cell and solid organ transplant patients, HCMV is a significant cause of morbidity and mortality [160].

HCMV is transmitted through body fluids: saliva, blood, urine, breast milk, as well as through sexual contact, organ transplantation and from mother to an unborn child (intrauterine infection)[160].

1.2.4 CLINICAL MANIFESTATIONS OF HCMV

HCMV infection in the immunocompetent host

HCMV may be acquired any time during life and the seroprevalence increases with age and is about 0.2-2,2% at birth, 40–60% in a middle age population and up to 90% in elderly[161]. The increased HCMV prevalence in the elderly may contribute to a general immunosuppression and increased incidence of different diseases, including cancer. Primary infection in immunocompetent individuals is usually asymptomatic, although mononucleosis like symptoms may occur, such as fatigue, malaise, fever, myalgias and headache. In some cases even hepatomegaly, splenomegaly and adenopathy may occur [162]. Very rarely HCMV infection is fatal in immunocompetent individuals, but that might be due to unknown immune defects or other undiagnosed illnesses[163].

In the recent decade much of epidemiological research suggests that HCMV may contribute to various diseases in immunocompetent hosts. For example, it has been shown that HCMV may be a contributing factor for development of cardiovascular disease and atherosclerosis[164, 165]. HCMV has been detected in bowel specimens of patients with inflammatory bowel disease[166]. The presence of HCMV was investigated in many other inflammatory diseases such as: rheumatoid arthritis, systemic lupus erythematosus, Sjögrens syndrome and psoriasis[167]. As HCMV is reactivated by inflammation, it has been debated whether the virus is an epiphenomenon or may be a contributing factor in the inflammatory process.

HCMV infection in immunocompromized hosts

In immunocompromized individuals such as AIDS patients, stem cell and solid organ transplant patients and in patients undergoing other immunosuppressive therapy (e.g. chemotherapy), HCMV infection is a significant cause of morbidity and mortality[168]. It can be caused by primary HCMV infection, reactivation of latent virus or reinfection with another HCMV strain. The severity of the HCMV infection depends on the level of immunosuppression of the host. Symptoms vary from almost asymptomatic to a severe end-organ disease, leading to high mortality. In addition, infection with HCMV in allogeneic stem

cell recipients and solid organ transplant recipients increases opportunistic fungal and bacterial infections [169]. In patients with hematopoietic stem cell transplantation, the main end-organ diseases caused by HCMV infection are pneumonia, gastroenteritis, retinitis and rarely also CNS involvement [170]. In order to lower the risks of HCMV infection that may lead to dangerous complications, hematopoietic stem cell transplant patients and solid organ transplant patients with high risk of HCMV infection receive antiviral treatment pre- and post-operatively[171].

In patients infected with the Human Immunodeficiency Virus HIV [172], a coinfection with HCMV is very common, reaching levels of 90%-100%. The amounts of HCMV-specific CD8+ T cells often exceed that of the HIV-specific T cells in patients with HIV[173]. In HIV patients, HCMV co-infection contributes to development of cardiovascular and cerebrovascular diseases[174]. Before the anti-retroviral therapy was available for HIV treatment, end-organ disease caused by HCMV was the most common manifestation of HIV infection, predominantly characterized by retinitis or gastroenteritis often occurring when CD4+ T cell counts drops below 50cells/mm^3. These manifestations are now much less frequent as antiretroviral therapy successfully preserves CD4+ counts [175], but the immune dysregulation associated with HIV-HCMV coinfection may be a future concern for survival.

Congenital HCMV infection

The incidence of congenital HCMV infection ranges from 0.2% to 5%[176]. Approximately 85-90% of infants with congenital HCMV infection are asymptomatic at birth. The rest 10-15% have some symptoms, manifesting as jaundice, petechial rash, hepatosplenomegaly and neurologic abnormalities such as microcephaly and lethargy. Ophthalmologic examination reveals chorioretinitis and optic atrophy in approximately 10% of symptomatic infants. Further in life the infants may develop sensorineural hearing loss and different levels of mental retardation [177]. One third of the symptomatic infants are born prematurely and approximately half of them are small for gestational age. The mortality rate in infants with congenital HCMV infection is <5% [178]

To date, congenital HCMV infection is the main cause of sensorineural hearing loss during childhood, leading to deafness in 10-15% of all infants with HCMV infection, of which 40–50% were born with symptomatic HCMV infection and 7–15% were asymptomatic at birth[176].

1.2.5 DIAGNOSIS OF HCMV

Clinical manifestation of HCMV is symptomatically similar to manifestations of other herpes viruses. Hence, laboratory testing is necessary for diagnosis. HCMV can be detected with the help of serology testing, IgG avidity, Quantitative Nucleic Acid Testing (QNAT), Antigenemia assay, virus culture, immunohistochemistry (IHC) and in situ hybridization (ISH).

Serology

Detection of HCMV specific antibodies indicates whether the patient has a primary infection or has been previously exposed to the virus. Detection of IgM antibodies usually demonstrates a recent infection, but due to low specificity of IgM antibody towards HCMV. When used IgM detection false-positive results may be misleading and additional serum tests should be followed over time. Detection of IgG antibodies demonstrates previous HCMV infection in individuals, while a more reliable test for acute HCMV infection is IgG avidity, which increases over time after infection[168, 179].

Virus culture

For a long time a virus culture assay was the golden standard method for detection of HCMV in clinical specimens. During recent decades new biological methods, such as QNAT, are gradually replacing HCMV virus culture assays as the primary diagnostic method for HCMV detection in clinical laboratories. Virus culture is demanding, time consuming and less sensitive than modern methods. It consists of co-culture of the clinical specimen with a fibroblast monolayer, followed by detection of early HCMV proteins with a monoclonal antibody. For faster detection of HCMV (within 24-48h) low intensity centrifugation can be used to enhance the infection of fibroblasts[180].

Quantitative nucleic acid testing (QNAT)

QNAT assays are gradually replacing antigenemia assay and virus culture because of their high specificity and sensitivity and quantitative read out, enabling the measurement of viral load in different specimens. Polymerase Chain Reaction (PCR) is the most commonly used method by clinical laboratories for HCMV detection [181]. PCR results may vary due to different techniques for DNA extraction and test assay design. In modern laboratories automatic sample processing is used in order to avoid the mismatch of the results [180, 182].

Antigenemia essay

In this assay a monoclonal antibody against pp65, one of the most abundant tegument proteins, is used. Pp65 is detected in polymorphonuclear cells in blood by direct immunostaining. The positive cells are then quantified and correlated to a threshold of HCMV load in the blood during infection. The threshold levels can vary according to clinical settings. This assay has been the most widely used method for HCMV diagnosis and for quantification of viral load in blood in the clinical setting since the 1990s, but has been gradually replaced by molecular biology methods. It has been proved to be more sensitive than culture of the virus from clinical specimens. It is rather simple, fast (one day) and cheap to perform. Commercially available kits have made it widely accessible in many clinics around the world[180].

Immunohistochemistry (IHC)

IHC of HCMV-infected tissues or blood cells using commercially available antibodies against various viral proteins permits direct imagining of proteins in various specimens. The main advantage of IHC is the possibility of visualization and co-localization of viral proteins with other cellular proteins or markers. The sensitivity and specificity of the antibodies used for IHC may vary greatly[183].

In situ hybridization (ISH)

This assay utilizes differently labeled (for example with fluorochrome) complimentary DNA and RNA strands for detection of HCMV nucleic acids in tissue specimens or cell preparations[184]. The main advantage of this technique is the localization and visualization of nucleic acids in the specimen[185].

1.2.6 TREATMENT OF HCMV

To date, the most common antiviral drugs used for treatment of established HCMV infection are ganciclovir, its oral prodrug valganciclovir, foscarnet, cidofovir and fomivirsen. In transplant patients high-dose acyclovir and valganciclovir have been used for prophylaxis, and GCV is used as standard treatment of active HCMV disease in clinical settings. All of the drugs except fomivirsen directly or indirectly target the viral DNA polymerase, while fomivirsen is an anti-sense oligonucleotide directed against the HCMV IE gene locus [186].

Ganciclovir (GCV)

GCV was the first antiviral agent to be approved for clinical treatment of HCMV infection and still remains the drug of choice for treatment of HCMV infections. GCV is an acyclic nucleoside analog of 2′-deoxyguanosine and is phosphorylated by the viral protein kinase UL97 and cellular kinases into a biologically active triphosphate form that inhibits viral DNA synthesis by acting as a nucleoside analogue to block the viral DNA polymerase[187]. Mutations for GCV resistance have been mapped both to the DNA polymerase UL54 and the viral protein kinase UL97 genes [188]. GCV is available in intravenous formulation and as a sustained-release intraocular implant approved for the treatment of HCMV retinitis. The main side effects of GCV treatment are hematologic abnormalities (primarily neutropenia, anemia, and thrombocytopenia) as well as kidney and liver toxicity[189].

Valganciclovir

Valganciclovir is the pro-drug of GCV. It has the same mechanism of action as GCV, although it is only available in enteral formulation. After intake, valganciclovir is rapidly metabolized to the active form in the intestinal wall and liver[190]. Valganciclovir, as GCV, has hematologic toxicity with neutropenia and anemia, but also diarrhea due to its direct effects on the intestine [182].

Foscarnet

Foscarnet is a pyrophosphate analogue, which inhibits viral DNA polymerase and does not require enzyme activation after intake[186]. Foscarnet is manufactured in intravenous formulation and is considered as a second-line therapy. Foscarnet is administrated when GCV therapy fails because of viral resistance, or for those who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia[191]. Because of its potential nephrotoxicity, the administration requires slow infusion, extensive prehydration, and frequent monitoring of serum creatinine levels [192]

Cidofovir

Cidofovir is an acyclic nucleoside phosphonate analogue. It is converted to the active form by cellular kinases and acts as inhibitor of viral DNA polymerase, causing premature chain termination in viral DNA synthesis [193]. Cidofovir is produced only in intravenous formulation due to its low oral bioavailability. The main advantage of Cidofovir is the long intracellular half time that allows effective treatments even by infrequent dosage[194]. The major limitations of Cidofovir are its side effects such as neutropenia and severe renal toxicity, leading to electrolyte imbalance. In preclinical studies cidofovir has shown carcinogenic and teratogenic effects[195]. Due to its profile of side effects, cidofovir is used only as a second line therapy.

Fomivirsen

Fomivirsen inhibits HCMV IE gene expression, which is vital for viral replication. It is produced in intravitreal formulation and is used in clinical settings for treating HCMV retinitis in HIV patients. Due to its intravitreal administration, it has no systemic effects during treatment. It has a half-life of approximately 55 hours, which allows infrequent dosage[196].

Novel anti-HCMV drugs

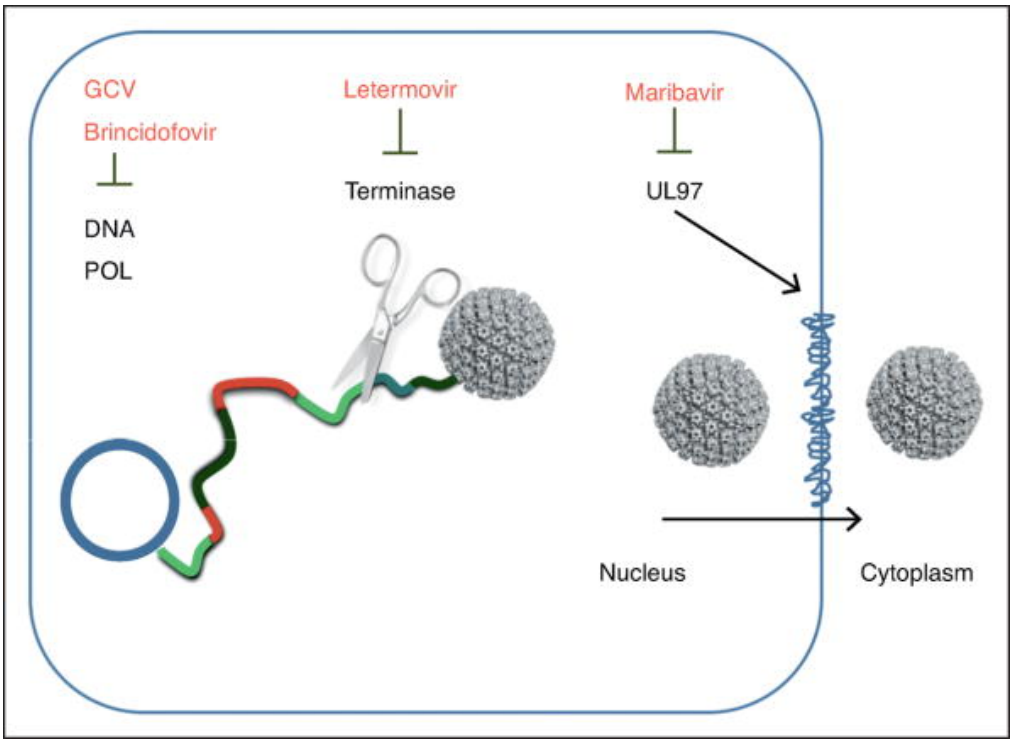
Most of anti-HCMV drugs have rather low bioavailability, long-term toxicity and teratogenic effects. Therefore there is a need for discovery of new antiviral components with alternative mechanisms and lower toxicity levels.

Currently three new antiviral drugs are undergoing preclinical studies with promising results. One of them is Maribavir, an inhibitor of the UL97 enzyme, which is necessary for viral egress from nucleus to the cytoplasm through the digestion of nuclear lamins (Fig. 5) [197]. Maribavir has undergone a successful phase II study and was further investigated in phase III randomized controlled trial, but failed to meet the primary endpoint: decreased end-organ disease. In that study the measurements of viral load were not taken in consideration, although it has been showed earlier that high viral load may cause end-organ disease [198, 199]. Due to its mechanism, Maribavir is contraindicated to be used in combination with

Ganciclovir as it blocks the initial phosphorylation of Ganciclovir, thus antagonizing the effect of the drug.

Another drug undergoing preclinical studies is Brincidofovir: a lipid prodrug of Cidofovir. It has no renal toxicity compared to the parent compound, although it possesses certain gastrointestinal toxicity (manifested as diarrhea), which is dose limiting (Fig. 5)[200]. Unfortunately, Brincidofovir failed the primary end-points of phase III study, which is to suppress the HCMV infection during 24 weeks after hematopoietic cell transplantation. The drug showed initial suppression of HCMV infection during first 10 weeks of the study, followed by 14 weeks of reactivation. In addition, significantly higher levels of graft vs. host disease were observed in the Brincidofovir treated group compared to placebo[201].

Letermovir is another antiviral drug underwent successful phase II trials. It has a distinct mechanism from most of the anti-HCMV drugs that mainly inhibit the DNA polymerase. Letermovir instead inhibits the viral terminase and final DNA packaging, which is needed for final cleavage of viral DNA prior to virus assembly and egress from the cell during lytic infection (Fig. 5)[202].



GCV-Ganciclovir

Figure 5. Mechanisms of novel antiviral drugs. *Four antiviral drugs act at different stages of HCMV virion production. Image modified from [203]*

Other anti-HCMV therapies

Another promising future treatment strategy against HCMV is immunotherapy. Today there are two types of immunoglobulin preparations (IVIG) for treatment of HCMV infection: hyperimmunoglobulins HCMV IVIG (purified IgG from HCMV seropositive individuals

with high IgG HCMV titers) and standard immunoglobulins polyvalent IVIG (from donor pools with unknown HCMV serological status)[204]. Initially, immunoglobulin prophylaxis was shown to lower HCMV infection and the mortality rate in bone marrow transplant patients, kidney transplant patients[205] and in cardiac patients with hypogammaglobulinemia[206]. More recent data have demonstrated that polyvalent IVIG (and not HCMV IVIG) have mainly reduced the incidence of interstitial pneumonitis but did not demonstrate the advantages obtained by other treatment. Additionally, treatment with polyvalent IVIG increased the risk for venous thrombosis, especially in the liver [207]. Because of such risk profile and other drawbacks of polyvalent IVIG treatment, such as risks for other diseases transmitted by blood-derived products and low amounts of effective antibody in the polyclonal preparations, it is not used in standard anti-HCMV treatment protocols[208]. It would be more beneficial for the patient to receive a more targeted therapy such as monoclonal antibodies against HCMV envelope glycoproteins, which would, for example, mediate neutralization, inhibit viral interaction with the cellular receptors, activate various cellular effector functions and induce anti-viral activity.

Another treatment strategy against HCMV is the development of neutralizing vaccines, but the understanding of the viral antigens important for synthesis of protective antibodies remains limited. It has been previously accepted that gB plays a crucial role during HCMV entry in the cells and therefore much of neutralizing activity of the host was directed against gB[209]. There is evidence that anti-gB antibodies reduce HCMV infection and recombinant gB vaccines decreased the risk of maternal and congenital HCMV infection[210] and reduced viremia[211]. Nevertheless, recent studies showed an increasing importance of the pentameric gH complex in cell tropism of HCMV, making it an attractive target in vaccine design. Pentameric gH complex is essential for viral infection of cell types directly linked to viral pathogenesis: endothelial cells, epithelial cells and leukocytes[172].

Despite the development of various immunotherapies against HCMV, the knowledge of protective HCMV immunity remains incomplete. In fact, preexistent HCMV immunity does not seem to prevent HCMV reinfection or reactivation of HCMV. The evidence for this statement is demonstrated by the fact that 30–60% of infants suffering from hearing loss caused by congenital HCMV infection are born from HCMV seropositive mothers[212, 213].

1.3 HCMV AND THE IMMUNE SYSTEM

HCMV has numerous mechanisms to subvert recognition by the host immune system. As mentioned earlier, HCMV produces more than 750 proteins, but just 50 proteins are essential for replication[130]. The remaining proteins are used for modifying the environment for HCMV in order to subvert the immune system of the host and remain undetected.

HCMV has been studied for decades and one of the most intriguing features is the ability to evade the immune response during its life in the host and remain undetected. The primary immune response exerted by the host is unable to prevent the virus from establishing latency. The subsequent long-term immune response is not able to clear the latent virus. HCMV interferes with a broad spectrum of immune responses starting with innate mechanisms such as inflammatory cytokines from virus- cell binding, natural killer cell induction, which then drives adaptive immunity with antibody production and the generation of specific CD4+ and CD8+ T cell responses. The immunomodulatory abilities of HCMV are summarized in figure 6.

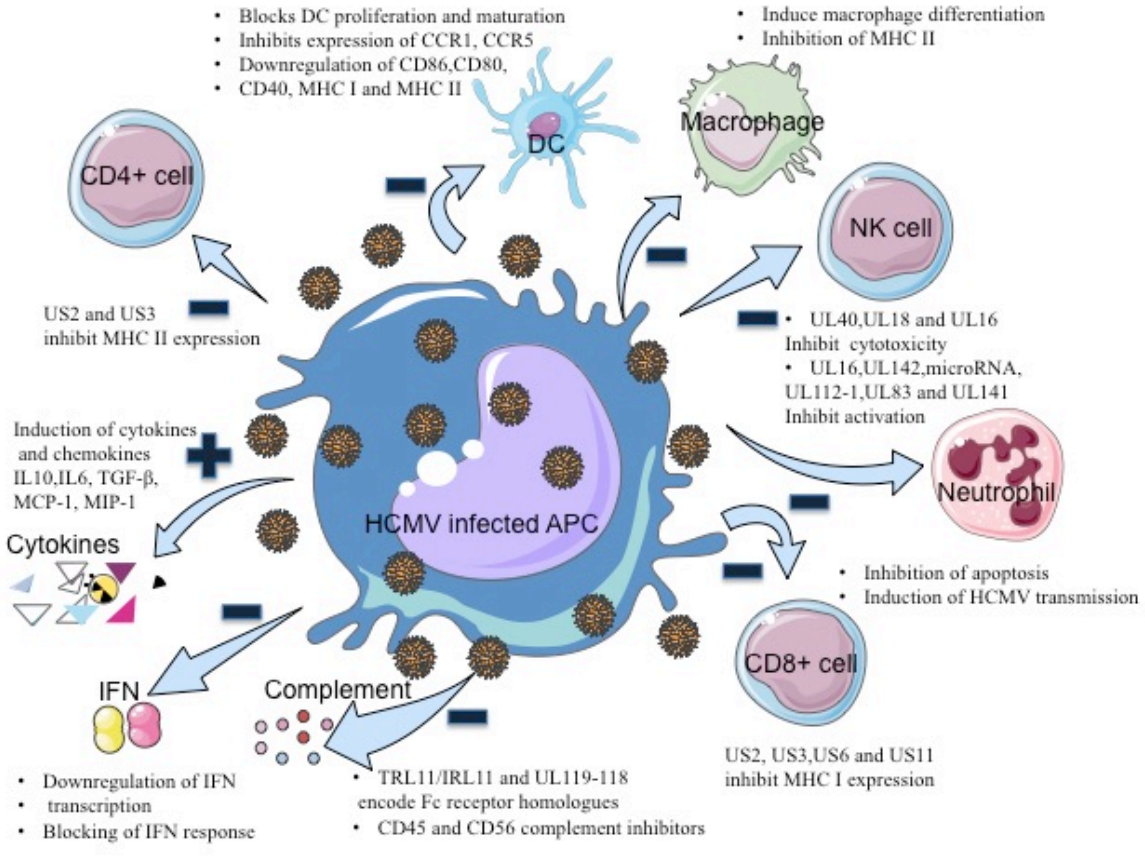


Figure 6. Summary of HCMV's immunomodulatory abilities

1.3.1 HCMV AND INNATE IMMUNITY

The innate immune response plays a critical role in the initiation of a primary immune response and further induction of adaptive immunity. The primary innate immune response to HCMV infection consists of interferons, recruitment and activation of antigen presenting cells (APC), phagocytes and natural killer (NK) cells. In a second step, the adaptive immune

system is activated to produce an antigen-specific immune response. HCMV has various strategies for interacting with innate immunity and they are discussed in detail in the following sections.

HCMV and NK cells

NK cells play a key role in innate immunity. They control viral infection of the early stages of infection and subsequently activate adaptive immunity. It has been described that NK cell defects may lead to severe HCMV infections[214]. HCMV glycoproteins (gB and gH) are recognized by Toll Like Receptors 2 (TLR) family and the subsequent binding of HCMV to TLR2 initiates production of proinflammatory cytokines, interferons resulting in activation of NK cells through upregulation of NFkB[215]. The importance of NK cells in anti-HCMV defense is indirectly suggested by the extensive mechanism that HCMV encodes to prevent NK cell activation. The viral protein UL40 induces HLA-E expression to inhibit functions of NK cells via the CD94/NKG2 inhibitory receptor[216, 217]. HCMV expresses a viral homologue of cellular MHC class I: UL18. It binds the inhibitory NK cell receptor LIR-1 and blocks NK cell activation [218]. In addition, five HCMV genes are known to prevent activating NK cell receptor signaling. UL142, UL16 and microRNA-UL112-1 interfere with NKG2D-mediated NK cell activation, UL83 interferes with NKp30 and UL141 with activation of CD226 and CD96 on NK cells[219]. HCMV UL16 protein also mediates resistance of infected cells to cytolytic proteins from NK cells [220].

HCMV and macrophages

Macrophages play an important role in initiating the innate immune response by presenting different antigens mainly to T cells and connect innate to adaptive immunity. It is well known that monocytes are not permissive for *de novo* viral gene expression and replication while, when differentiated into macrophages, these processes are permitted[221-223]. HCMV "solves" this problem by prolonging the life of infected monocytes and inducing their differentiation into macrophages by involving the caspase-3 pathway, because macrophages support viral replication and the release of infectious virions [224, 225]. The release of produced infectious virions by HCMV-infected newly differentiated tissue macrophages would lead to a long-term infection within peripheral organ tissues, which may explain organ pathology observed in immunocompromized hosts. Additionally, HCMV is able to modulate the immune response by choosing which antigens and epitopes to target. Therefore HCMV interferes with antigen presentation on MHC II in macrophages and subsequently inhibits further T cell proliferation[226].

Interleukine-10 (IL-10) is a suppressive cytokine, with multiple immunosuppressive effects in both innate and adaptive immunity. HCMV produces an IL-10 homologue (discussed in depth later in this chapter) that is believed to interfere with DC, microglial and macrophage functions through interference with normal differentiation and cytokine production through inhibition of NF-κB[227]. In addition, during HCMV infection of monocytes/macrophages, increased levels of proinflammatory cytokines such as IL-1, IL-6 and TNF-α are observed.

This proinflammatory ability of HCMV may contribute to an inflammatory environment and has been linked to oncogenic transformation in inflammation-induced animal models of cancer[82].

HCMV and Dendritic cells (DC)

DC are very potent antigen presenting cells (APC) and are central in initiation and maintenance of adaptive immune response by activating T and B cells, as well as by inducing cytokine and chemokine production. DCs are the front line of defense and, naturally, many pathogens, including HCMV, have built various mechanisms for blocking them. HCMV targets DCs, by blocking their proliferation and maturation. The virus paralyzes DCs, making them unable to present HCMV antigens and further activate T cells[228, 229]. Additionally, HCMV downregulates cell surface expression of important T cell costimulatory molecules such as CD86, CD80 and CD40, as well as MHC class I during early infection and MHC class II at later stages of infection[230, 231]. HCMV also inhibits expression of CCR1 and CCR5, which are important chemokines for DC migration to sites of inflammation, thereby preventing DC migration[232]. The HCMV IL-10 homologue also inhibits the maturation of DCs and alters their functions, leading to decreased cytokine production (ex. IL-12), impaired T cell proliferation and reduction of multiple co-stimulatory molecules on DC[233].

HCMV and neutrophils

Neutrophils also play an important role in innate immunity. It has been demonstrated that HCMV infection inhibits apoptosis of neutrophils and makes them more effective in performing effector functions such as phagocytosis of bacteria[234]. Prolonged life span of neutrophils by HCMV could further result in accumulation of over-reactive neutrophils in the tissues, potentially leading to tissue damage. HCMV-infected endothelial cells induce chemotaxis of neutrophils by the secretion of neutrophil chemoattractants C-X-C chemokines, IL-8 and GROα. Infected endothelial cells seem to be able to transmit the virus to neutrophils through direct cell-to-cell contact during neutrophil transendothelial migration[235]. This transmission of HCMV to neutrophils further increases viral dissemination in the body.

HCMV and the complement cascade

Antibody mediated complement lysis is an important mechanism for elimination of HCMV infected cells. HCMV encodes complement regulatory proteins in order to escape complement mediated cell lysis. HCMV TRL11/IRL11 and UL119-118 encode Fc receptor homologues, thereby hiding the virus from complement binding[236]. Another viral mechanism of interference with the complement-mediated cell lysis is the expression of surface complement inhibitors CD35, CD46 and CD55[237].

HCMV and interferon (IFN) signaling

Another part of the innate immunity that is modulated by HCMV is the production and signaling induced by interferons. The type I IFNs, IFNα and IFNβ are crucial for building the innate immune response to viral infections. The binding of IFNα and IFNβ to the IFN receptor induces Jak/Stat signaling leading to the rapid upregulation of interferon-stimulated genes, such as MHC class I molecules and various cytokines[238]. HCMV's proteins IE1 and IE2 interfere with IFN signaling by downregulation of their transcription[239, 240]. Additionally, HCMV's tegument protein pp65 blocks IFN response by blocking the interferon response factor 3 (IRF3) activation[241]. This modulation of innate the immune response permits the virus to be undetected in the host for longer time during the initial phase of the infection.

HCMV cytokine homologue

Interleukin-10 (IL-10) is an immunosuppressive cytokine and HCMV produces an IL-10 homologue which blocks expression of proinflammatory cytokines such as IFN-γ and TNF-α, inhibits expression of MHC I and MHC II on PBMCs and DC and hinders proliferation of PBMCs[242]. An alternative splice version of HCMV IL-10 homologue is also produced during latency. Although the IL-10 latency associated homologue fails to fully activate an IL-10 signaling pathway, it decreases the expression of MHC II on granulocyte-macrophage progenitor cells and monocytes limiting the clearance of latently HCMV-infected cells[155].

HCMV and γδ T cells

γδ T cells belong to a subtype of T cells bridging innate and adaptive immunity and represent only 1-10% of the whole T cell population. They are subdivided in two groups based on their TCR chain: Vδ1 and Vδ2 T cells. Vδ2 T cells represent the largest group in blood, consisting of up to 95% of all γδ T cells, while in tissues the vast majority of γδ T cells have the Vδ1 TCR. [243]. Although the γδ T cell population is small, during bacterial or viral infection an impressive increase from 1% up to 50% may be observed in the blood [244]. Dendritic cells play an important role in activation and proliferation of γδ T cells, enhancing their cytotoxic and immunoregulatory functions. Furthermore, γδ T cells can activate NK cells, Th1 and CD8+ cytotoxic T cells and even induce direct tumor killing through various cytokines and chemokines[245].

γδ T cells are not MHC restricted and therefore not affected by HCMV's inhibition of HLA molecules and recognize self-antigens on the surface of stressed cells, such as virus infected cells or tumor cells [246, 247].

In kidney, lung and stem cell transplant recipients HCMV infection induces expansion of γδ T cells. The exact interplay and pathway by which HCMV induces γδ T cells expansion remains to be elucidated[219, 248]. Nevertheless, γδ T cell expansion in transplant patients is associated with infection resolution[249] and tissue-associated Vδ2-negative γδ T cells show

a cytotoxic effector memory phenotype upon HCMV challenge[250], indicating that these cells play an important role in mounting an anti-HCMV response.

HCMV and apoptosis

Apoptosis or programmed cell death is an important mechanism for elimination of virus-infected cells as a part of host innate immunity. In order to survive in the host HCMV has developed mechanisms to also avoid apoptosis of the infected cells. HCMV encodes two major proteins that directly interfere with apoptosis. The HCMV UL36 gene encodes a viral inhibitor of caspase-8-induced apoptosis and exon 1 of UL37 encodes the viral mitochondrial inhibitor of apoptosis[251].

1.3.2 HCMV AND ADAPTIVE IMMUNITY

HCMV and T cell activation

HCMV has multiple mechanisms for interfering with T cell activation. Many of them interfere with antigen presentation on MHC I and II class complexes. Phosphorylation of IE-1 by pp65 blocks the processing of IE-1 in the proteasome and further presentation on APC[252]. Additionally, products of IE and E HCMV genes downregulate MHC I and II class complexes thus blocking the presentation of HCMV antigens on APCs and the further activation of T cells [253]. Furthermore, HCMV encodes four genes (US2, US3, US6 and US11) whose protein products directly interfere with the processing of MHC class I, US2 and US11 are involved in the degradation of newly synthesized MHC class I molecules[254]. US3 retains MHC class I peptide complexes in the endoplasmic reticulum [255]. The product of viral gene US6 blocks peptide translocation into the endoplasmic reticulum [256]. Moreover, US2 and US3 help HCMV to evade the immune response by decreasing the surface expression of MHC class II. Additionally, MHC class II is also downregulated by HCMV pp65 protein[257].

HCMV and CD4+T cells

CD4+ T cells have various functions. T helper cells are important activators of CD8+ cytotoxic T cells (Th1 cells) and of B cells (Th2 cells). Th17 cells are important in inflammation and anti-parasite responses. Regulatory T (Treg) cells have immune suppressive abilities. All these cells produce various cytokines: Th1 cells mainly produce pro-inflammatory cytokines: IL-2, IFN-γ, TNF-α while Th2 cells mainly produce anti-inflammatory cytokines: IL-4, IL-5, IL6, TGF-β and IL-10. In addition, the immunosuppressive cytokines TGF-β and IL-10 are also secreted by Tregs. In order to create a suitable environment for viral persistence, HCMV induces production of various cytokines and chemokines including IL-6, TGF-β, IFN-γ, TNF-α, MCP-1, MIP-1 and others [219]. In order to further induce local inflammation, HCMV encodes four chemokine receptor homologues: UL33, UL78, UL27 and UL28[167]. In order to avoid recognition by CD4+ T cells and further activation of an adaptive immune response, HCMV downregulates MHC class II complex[253]. Similar to CD8+ T cells, most of the CD4+ T cells in seropositive

individuals are pp65 and IE specific[258] and approximately 10% of the total peripheral CD4+ T cell pool is HCMV-specific[259]. In transplant patients, it has been observed that IFN-γ and TNF-α, both produced by HCMV specific Th1, are important for CD8+ T cell control of HCMV[219]. To respond to this anti-HCMV activity, the virus reduces expression of TNF-α receptors on the cell surface[260].

HCMV and CD8+ cells

During viral infection, CD8+ cytotoxic T cells have an important role of limiting the HCMV infection by killing the infected cells. A strong correlation is shown in hematopoetic stem cell transplant patients between CD8+ population recovery and protection of HCMV infection. For example patients receiving *ex vivo* expanded HCMV CD8+ T cells are protected from primary and reactivated HCMV infection [261, 262]. Most seropositive individuals have a very strong CD8+ T cell response against viral pp65 and IE proteins[219]. Rapid expansion of pp65- and IE-specific CD8+ T cells is observed during primary infection, followed by formation of memory T cells. 10% of peripheral CD8+ memory T cells are directed against HCMV and this percentage increases with age[219]. HCMV specific CD8+ memory T cells do not express chemokine receptors, making it impossible for them to adequately react to inflammatory signals and migrate to the sites of inflammation. These cells are therefore found mainly in the bone marrow and not in the blood[263]. In order to avoid recognition by CD8+ cells US2, US3, US6, US11 gene products from HCMV downregulate MHC class I complex[264].

Antibody responses against HCMV

The production of antibodies during humoral response is an important part of the adaptive immunity. Neutralizing antibodies are produced against both structural and non-structural proteins and are important for host protection and inhibition of viral spread. HCMV's tegument proteins pp150, pp28 and pp65 are known to be highly immunogenic. Antibodies against pp150 are present in nearly all seropositive individuals. Pp65 induces strong antibody response during the acute phase of infection and it subsides shortly thereafter[265]. In the past, it was suggested that virus neutralization was mainly mediated by gB and gH[266, 267]. Recent studies show that antibodies against the pentamer complex gH/gL/UL128-131 that mediates viral entry into endothelial, epithelial and myeloid cells, are of greater importance[268].

1.3.3 HCMV AND IMMUNOSENESCENCE

Immunosenescence is the process describing immune aging. It is defined as age-associated changes in the immune system that lead to gradual dysfunction or dysregulation of innate and adaptive immunity[269]. It is unclear how this immune phenotype develops. Emerging evidence suggests that immunosenescence is not only dependent on human chronologic ageing, but it may reflect an exhaustion of the immune system. This exhaustion may be caused by processes that constantly activate the immune system, such as inflammatory diseases, virus infections (for ex. HIV and HCMV), cancer and organ transplantation [270,

271]. To keep control of latent HCMV infection and reactivated HCMV infection is a demanding task for the immune system. Although a direct link between HCMV and immunosenescence remains controversial, HCMV affects the immune system in various ways, some of which are similar to the natural process of immunosenescence and others may gradually lead to immunosenescence. For example, frequent reactivations of HCMV may "overstimulate" and exhaust the immune system. Additionally, latency-associated HCMV proteins, which constantly stimulate the immune system of the host, may also lead to phenotypical exhaustion and further immunosenescence. These aspects will be further discussed below.

HCMV and antigen stimulation

Recent studies show that chronic antigen stimulation may induce immunosenescence[272, 273]. HCMV infection is considered a major contributing factor to chronic antigen stimulation. HCMV induces accumulation of terminally differentiated T cells, which may contribute to both biological aging and immune ageing[272, 273]. The age related alterations in the immune system affect both the innate and adaptive parts. These age-associated changes lead to reduced lymphocyte production and function, decreased T cell activation and proliferation of both T helper cells and cytotoxic T cells. They also lead to a decrease in the naïve T-cell pool and expansion of memory T-cell pool and phenotypical changes of NK cells, resulting in a decline of cytotoxicity and cytokine production[273].

HCMV and memory inflation

During persistent HCMV infection, the memory T-cell pool appears to be shifted toward greater abundance of cells with a more restricted repertoire. This process is referred to as memory inflation. During memory inflation the memory T-cell pool expands, while the naïve T-cell pool decreases. Memory inflation appears to be caused by repeated T-cell stimulation and keeps latent infections under control. In the case of HCMV, up to 50% of the T-cell pool can be active against a single viral epitope[259, 272]. If a new pathogen challenges the immune system, the T-cell response may be limited due to the restricted T cell diversity. The diversity of T cell repertoire is restricted because the memory HCMV T cells are becoming dominant in the T cell pool when activated with a previously encountered antigen[274]. Memory inflation leads to accumulation of terminally differentiated T cells, which may cause both premature aging of the immune system and biological aging of the individual.

HCMV and CD28 expression in T cells

CD28 is an important co-stimulatory molecule expressed on T cells. It has been suggested that CD28 is essential for T-cell activation, survival, production of inflammatory factors and clonal expansion [275]. Thus, loss of the CD28 molecule on T cells may have negative consequences for the immunity of the host. Additionally, CD28 loss on the T lymphocytes is considered to be one of the signs of immunosenescence. Although $CD4^+CD28^-$ T cells are thought to be terminally differentiated, they show high immunological activity. $CD4^+CD28^-$

T cells produce the cytotoxic molecules perforin and granzyme B as well as TNFα, IFNγ, and IL-2 and also express NK cell receptors (KIR2DS2, NKG2D, CD11b, CD161)[276, 277]. Recent studies demonstrated that the loss of co-stimulatory molecules CD28 and CD27 on T cells occurs at different stages of HCMV infection: early, $CD8^+CD27^+CD28^+$; intermediate, $CD8^+CD27^+CD28^-$; and late, $CD8^+CD27^-CD28^-$ [278, 279]. This suggests a possible role of HCMV in the loss of the CD28 co-stimulatory molecule and in the consequent development of immunosenescence. In fact, expansion of the CD4+CD28- cell population has been observed only in HCMV seropositive individuals and these cells are a hallmark of immunosenescence[280]. Differentiation of CD4 T cells into $CD28^-$ T cells may depend on the presence of plasmacytoid dendritic cells and their production of IFN-α and TNF-α that are pro-inflammatory cytokines important for HCMV replication and viral survival in the host [281]. Additionally, it has been demonstrated that CD4+CD28- cells themselves may produce IFN-γ and even TNF-α [281, 282] in order to ensure expansion of CD4+CD28- cell population. We could speculate that CD4+CD28- expansion may be needed for the viral survival and not for controlling the virus as IFN-γ and TNF-α drive HCMV replication and reactivation in myeloid cells.

Role of HCMV in expansion of CD57+CD28- T cell population

Expression of the CD57 protein has also been associated with immunosenescence. Initially, CD57 was found on NK cells, but further studies demonstrated its presence on T lymphocytes and cells of neural crest origin[283]. CD57 has been considered to be a marker of immunosenescence in terminally differentiated T cells[284].

It has been shown that HCMV infection also has an ability to expand the $CD8^+CD57^+CD28^-$ cell population[280]. Since the loss of CD28 was associated with aging of the immune system and $CD28^-$ T cells were mostly HCMV-specific oligoclonal cells, $CD8^+CD57^+CD28^-$ T cells were also believed to be dysfunctional and senescent. However, recent studies show that these cells have polyfunctional abilities. For example, they respond through cytokine production to staphylococcal enterotoxin B stimulation[285]. At first, $CD57^+CD27^-CD28^-$ T cells were described as a population of cells that expanded only during acute HCMV infection[286, 287]. But recently it has been demonstrated that $CD57^+CD27^-CD28^-$ cells are maintained at high levels after resolution of an acute phase of HCMV infection [280]. It has also been suggested that latent HCMV infection may also increase $CD57^+CD27^-CD28^-$ cell populations[273].

Despite controversy on the direct involvement of HCMV in immunosenescene, it is well accepted that HCMV affects the immune system in numerous ways. Immunological alterations induced by HCMV infection lead to a certain immunological phenotype which may in turn lead to gradual immunosenescence observed in the elderly and in patients with inflammatory diseases and cancer. Although some effects of HCMV infection affecting immune system may be obvious, others are indirect and create uncertainty on whether HCMV-induced immune phenotype allows for disease progression or if HCMV is directly

contributing to the pathogenesis of different diseases. For example, do HCMV-infected cancer patients with immunoscenescencent phenotype have a worse prognosis due to this immune phenotype or because the virus drives cancer progression or immunosenescence? Consequently, it is not completely clear what role HCMV has in cancer development. Whether immunosenescence is a pathological immune phenotype of HCMV-infected individuals or whether the changes in immunological phenotype are only an epiphenomenon of infection and simply reflecting the activity of the HCMV infection in the individual.

1.4 HCMV AND CANCER

1.4.1 PRESENCE OF HCMV IN DIFFERENT TUMORS

Accumulating evidence suggests a link between persistent HCMV infection and cancer[288, 289]. Although the role of HCMV in cancer is still debated, HCMV proteins and nucleic acids are frequently detected in different types of cancers such as breast[290], colorectal[291], prostate[292], mucoepidermoid salivary gland tumors[293], medulloblastoma, glioblastoma[114, 117, 294], neuroblastoma[123] and rhabdomyosarcoma[295]. HCMV is also present in 94-98% of sentinel lymph nodes[296] and brain metastasis of patients with primary breast and colorectal cancers[297] while HCMV infection is absent in healthy surrounding tissues. The presence of HCMV only in tumor cells may suggest a strong correlation between an active HCMV infection and the development of cancer. Viral proteins and nucleic acids are mainly detected in tumor cells, endothelial or inflammatory cells within the tumor, but, so far, infectious virus has not been recovered from primary tumors. In earlier studies, it has been demonstrated that the level of HCMV infection in GBM tumors has a prognostic value and that patients with higher levels of HCMV infection in GBM tumors have shorter overall survival [118]. Therefore, the main challenge is to understand whether HCMV is just an epiphenomenon or is it a contributing or even initiating factor in cancer development.

1.4.2 ONCOMODULATORY ABILITIES OF HCMV

Healthy cells may evolve progressively to a neoplastic state. They obtain certain properties and capabilities in a complicated multistep pathogenic process that leads to cellular phenotypical changes and further development of malignant tumors. Malignant tumors are able to metastasize in the body, spreading through the blood and lymphatic system, which often makes them incurable. Increasing evidence suggests that various infectious agents may cause human cancer. Different viruses have been suggested as promoters of neoplastic transformation and are associated with various cancer forms. For example, Hepatitis C virus chronically infects hepatocytes and causes chronic inflammation resulting in hepatocellular carcinoma[298]. Similarly, infection with hepatitis B virus is considered one of the major causes of hepatocellular carcinoma[299]. Epstein Barr Virus (EBV) is believed to be associated with the development of nasopharyngeal carcinoma in individuals exposed to certain environmental carcinogens[300]. Human Papilloma Virus (HPV) has also oncogenic properties and has been associated with cervix cancer and oropharyngeal cancer[301]. HIV is associated with Kaposi's sarcoma, non-Hodgkin's lymphoma, Hodgkin's lymphoma and cancers of cervix, anus and conjunctiva[302]. Another oncovirus is human T-cell leukemia retrovirus (HTLV) that is associated with T cell leukemia[303]. Human Herpes virus-8 is believed to be the cause of Kaposi's sarcoma[304]. Merkel cell polyoma virus is associated with Merkel cell carcinoma, an aggressive cutaneous cancer form[305]. Although HCMV has not been directly associated with the neoplastic transformation, it has been proposed that persistent HCMV infection may contribute to development of cancer. Hence a term "oncomodulation" has been proposed to describe the role of HCMV in carcinogenesis. Oncomodulation implies that HCMV infects tumor cells and modulate their malignant properties but it is unable to transform normal cells into cancer cells[288, 289]. It has been proposed that viral proteins affect cell cycle progression differently in different cells,

depending on the cellular differentiation level. This would promote cellular growth in tumor cells and not affect the surrounding non-tumor cells [306, 307]. Frequently, a chronic inflammation in malignancies plays a crucial role in the shift from neoplastic precursor to invasive malignancy. The inflammatory process is considered the hallmark of neoplasia[308, 309]. Although the role of HCMV in cancer has been debated, the growing evidence that HCMV's proteins are present in various tumors raises a question whether HCMV infection could induce or contribute to the inflammatory process and to carcinogenesis. In fact, many of the biological responses caused by HCMV infection are similar to the ones that support chronic inflammation. The oncomodulatory abilities of HCMV are summarized in figure 7 and will be further discussed below.

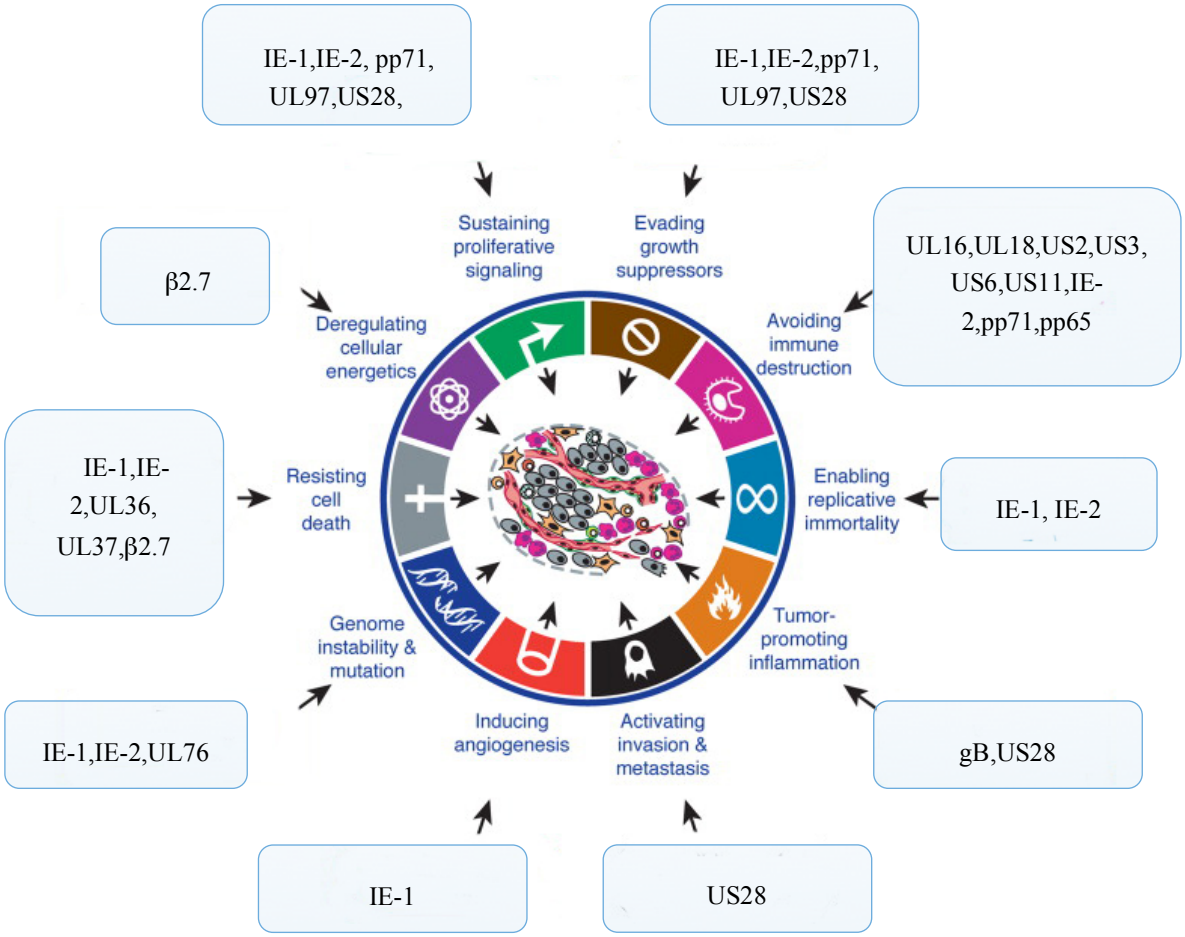


Figure 7. Summary of HCMV viral genes, inducing "the hallmarks of cancer". Modified from [310]

Evasion of apoptosis

Apoptosis is the programmed cell death and is essential during embryonic development and for the maintenance of tissue homeostasis. Apoptosis is also important as an innate defense mechanism for elimination of pathological cells. Two separate pathways have been described for the initiation of apoptosis: intrinsic and extrinsic pathways. Both of them lead to caspase activation and induction of cell death[311]. The intrinsic pathway is regulated by the Bcl-2 family proteins and activated by signals resulting from DNA damage and other cellular stress. Many other proteins can regulate the intrinsic pathway, for example p53 and Rb proteins, which are produced in response to DNA damage[311]. The extrinsic pathway begins outside

the cell and is activated through cell death receptors, such as FAS ligand receptor and the TNF-α receptor.

HCMV has developed several mechanisms for inhibition of apoptosis. Several HCMV gene products have distinct apoptotic properties, which inhibit cell death and enhance the survival of HCMV infected cells. HCMV proteins IE-1 and IE-2 block apoptosis mediated by TNF-α [312]. Additionally, it has been observed that IE-2 protein binds to p53 and directly inhibits its activation[313]. Other HCMV proteins such as UL36 and UL37 block apoptosis. UL36 inhibits caspase activation, thus blocking Fas-mediated apoptosis[314], while UL37 inhibits the mitochondrial pro-apoptotic proteins Bax and Bac resulting in inhibition of mitochondria-mediated apoptosis[315]. Some studies show the HCMV non-coding RNA β2.7 inhibits apoptosis in infected glioma cells through stabilization of the mitochondrial respiratory chain complex I[316]. In addition it has been shown that HCMV proteins may induce the expression of Blc-2 and COX-2 during persistent HCMV infection in colon cancer cells, resulting in resistance to cytotoxic drugs[291].

Sustained proliferation

One of the most important properties of cancer is the ability to sustain constant proliferation. In normal cells the proliferative process is strictly controlled by complex signaling pathways that transduce various signals depending on the cellular environment. These signals, which decide the fate of the cells, include growth factors and cytokines. In cancer cells, these processes are deregulated. This leads to sustained cell proliferation and tumor growth. In tumors, two major signaling pathways are frequently mutated: the MAPK pathway and the PI3K/AKT pathway. Mutation or induced activation in these pathways is shown to stimulate the cell growth, proliferation and survival[317, 318].

HCMV has multiple mechanisms to promote cell proliferation. It has been demonstrated that HCMV activates the PI3K pathway in human fibroblasts. Additionally, PI3K pathway activation is important for initiation of viral DNA replication [319]. Some studies demonstrated that HCMV has multiple mechanisms to activate MAPK, an important kinase for viral replication[320]. Other studies show that HCMV IE-1 protein blocks Rb by promoting its phosphorylation and decreases the expression levels of p53 in HCMV infected GBM cells[307]. These data indicates that HCMV IE-1 may have an oncogenic potential and affect proliferation and survival of HCMV infected GBM cells.

Additionally, HCMV encodes for chemokine receptor homologue, HCMV US28, which is important in tumor development as it induces activation of pathways involving STAT-3, IL-6, vascular endothelial growth factor (VEGF) and cyclooxygenase-2 (COX-2) in US28 positive cells [321-323]. Several HCMV regulatory proteins, such as IE-1, IE-1 and the tegument proteins pp71 and UL97, are shown to inactivate proteins of pRb family and thus promote the entry of infected cells into the S phase of the cell cycle [324-326]. Additionally, HCMV proteins IE-1 and IE-2 may deregulate cell cycle checkpoint controls by interacting with p53 suppressor proteins [326].

Limitless replicative capacity

Healthy cells have a lifetime with a certain amount of expected cell divisions. Thereafter they undergo senescence and die. This natural process is controlled by multiple mechanisms, but it may be circumvented by blocking the pRb and p53 tumor suppressor proteins, which will allow endless cell divisions- a process termed immortalization[327]. Tumors frequently express telomerase, an enzyme that protects the telomeres from shortening. This gives the cancer cell endless replicative ability and prevent cellular senescence[328].

It has been demonstrated that the HCMV IE-1 protein induces telomerase activity and telomere lengthening through interaction with the hTERT promoter in HCMV infected GBM cells. Furthermore, IE-1 and hTERT proteins are co-expressed in cells in GBM tissue samples[329]. HCMV expresses two proteins, pp71 and UL97, that are involved in phosphorylation and inactivation of the Rb family tumor suppressor proteins [324, 325], thereby modulating typical oncogenic features in the infected cells such as proliferation and survival.

Genomic instability

Genomic instability is another characteristic of cancer cells. During limitless divisions a destabilization of the cancer cell genome occurs. This is considered to be a major driving force for tumorigenesis. It has been demonstrated that mutations and genomic instability may contribute to tumor progression. For example, genetic instability may lead to various forms of mutations in important cellular genes: amplifications, deletions, rearrangements of chromosome segments, gain or loss of entire chromosomes and others. Specifically, mutations in p53, pRb and Bcl-2 genes are proposed to be responsible for driving several hallmarks of cancer[330], and HCMV interferes with these genes as mentioned earlier.

Some studies have shown that HCMV can induce genetic instability by stimulating DNA breakage, for example in chromosome 1 in HCMV-infected cells[331]. Additionally, deletion of 1q42 chromosome has been associated with the development of GBM[332].

It has been proposed that the HCMV protein UL76 can induce micronuclei, misalignment of chromosomes, lagging and bridging thereby inducing DNA damage[333]. The HCMV proteins IE-1 and IE-2 together with adenovirus E1A protein induce mutations in the p53 gene as a part of cell transformation of baby rat kidney cells[334]. The transformed cells had a mutated p53 gene, which may be one of the mechanisms by which HCMV IE proteins contribute to transformation. However, HCMV was not detectable in transformed cells, implying that HCMV may cause transformation by a “hit and run” mechanism[334].

Angiogenesis

Another important component in tumor development is the induced formation of new blood vessels: angiogenesis. With the growth of the tumor mass, the need of vascularization is constantly increasing. In the tumor, the balance between pro-angiogenic (for ex. VEGF and

basic fibroblast growth factor) and anti-angiogenic factors (for ex. thrombospondin-1) is shifted and the formation of new blood vessels is induced. This has a great impact on tumor progression and metastasis[310].

Several studies have demonstrated that HCMV has an ability to promote angiogenesis. HCMV's gene product US28 induces angiogenesis by upregulation of VEGF. In HCMV infected GBM cells, US28 contributes to a proangiogenic phenotype through increased VEGF production[323]. In addition it has been demonstrated that HCMV downregulates the expression of trombospondins, which are essential for angiogenic control in HCMV-infected human gliomas and human retinal glial cells[335]. It has been proposed that HCMV glycoprotein B (gB) can directly bind and activate platelet derived growth factor (PDGF-α) in GBM cells, resulting in phosphorylation of PDGF-α, which in turn promotes migration and angiogenesis in GBM[144]. Additionally, it has been proposed that HCMV-mediated activation of COX-2 may also promote angiogenesis in tumor cells since COX-2 induces the expression of VEGF, bFGF, PDGF, iNOS, and TGF-α in tumor cells. These molecules are able to promote capillary endothelial cell migration[81].

Epigenetic changes

Epigenetic changes are mechanisms that regulate gene expression without changing the underlying DNA sequence and have an important role in cancer. For example DNA methylations have been proposed to play a major role in cancer development. Studies have demonstrated that hypermethylations of tumor suppressor genes as well as genome wide hypomethylations are frequently present in many cancer types and would affect gene expression[336]. HCMV can interfere with DNA methylation during viral infection, and it has been demonstrated that epigenetic mechanisms play an important role in the viral life cycle[337]. It has been shown that HCMV infection can cause a global hypomethylation in fibroblasts. Further investigations revealed that DNA methyltransferases are delocalized from the nucleus to the cytoplasm in HCMV infected cells[338]. These findings indicate that epigenetic regulatory mechanisms may be essential for viral interaction with the genome of the infected cells. These epigenetic changes, induced by the virus may contribute to impairment of protective mechanisms of the infected cell as well as may give an opportunity to the virus to replicate its genome in the infected cell.

2 AIMS OF THE THESIS

- To investigate the ability of HCMV to subvert the immune system affecting proliferation of CD4+ T cells.

- To study the ability of HCMV to induce a more aggressive CSC-like phenotype in primary GBM cell lines.

- To examine the T cell phenotype in the blood of HCMV-infected GBM patients and whether a specific T cell phenotype has a prognostic impact value.

- To investigate the immune response to HCMV in GBM patients.

- To examine cytokine patterns in the blood of GBM patients and investigate whether neutrophil activation is associated with HCMV infection and GBM progression

3 RESULTS AND DISCUSSION

3.1 HUMAN CYTOMEGALOVIRUS PARTICLES DIRECTLY SUPPRESS CD4 T-LYMPHOCYTE ACTIVATION AND PROLIFERATION. (PAPER 1)

CD4+ T cells are crucial for the establishment of a strong and well-organized immune response and are important players in the formation of adaptive and humoral immunity. Thus, these cells are essential in combating viral and bacterial infections. HCMV is a pathogen with the ability to subvert the immune responses and is therefore able to coexist with the host and establish latency. Development of a well-established CD4 response against HCMV is associated with asymptomatic HCMV infection[339].

In this study, we have examined the ability of HCMV to directly affect the proliferation of CD4+ T cells, which could be another viral mechanism to weaken and mislead the immune system. In this paper we, showed that proliferation of T cells obtained from PBMC preparations of healthy donors is directly inhibited by HCMV. Since PBMCs also contain monocytes, DC and other lymphocytes, which could affect CD4+ T cell proliferation, we purified CD4+ T cells from healthy donors in order to further study direct effects of HCMV on the CD4+ T cell population. When the CD4+ T cells were stimulated with Phytohemagglutinin (PHA) in the presence of HCMV, T cell proliferation was blocked. T cell proliferation was unaffected when exposed to filtered viral inoculums or to viral supernatants obtained after ultracentrifugation of viral stocks, indicating the importance of viral particles, and not soluble factors in this inhibitory process. HCMV directly inhibited the proliferation of PHA-stimulated CD4+ T cells in a dose dependent manner, with higher inhibition observed at higher multiplicity of infection (MOI). Other herpes viruses (I and II) and measles virus did not affect CD4+ T cell growth, suggesting that while HCMV possesses this ability, it is not a general phenomenon among viruses. HCMV blocked T cell proliferation even when T cells were stimulated with other mitogens such as Concavallin A (ConA), phorbol myristate acetage (PMA) or with IL-2, indicating that HCMV is capable of inhibiting T cells, regardless of the activation pathway. The inhibition of proliferation was neither affected by washing of the T cells after infection nor by co-stimulation with IL-2.

During proliferation and activation, CD4+ T cells express different activation markers, the most abundant being CD69 and CD45RO. It has been proposed that HCMV may induce some changes in the CD4+ T cell phenotype during viral infection. During the peak of HCMV infection, HCMV-specific CD4+ T cells are CD45RA/CD45RO double positive, and express CD27, CD28, CD38, and CD40L[340, 341]. We showed that regardless of the fact that the proliferative ability of the T cells was impaired by HCMV, the expression of CD69 and CD45RO was not affected. However, HCMV blocked T cell production of several cytokines, including TNF-α, IFN-γ and IL-4. This suggests that HCMV is able to modify the immune response by affecting T cell proliferation in order to allow its persistence through hiding from the immune system. The above observation together with the one that HCMV inhibits proliferation of CD4+ T cells and makes them unresponsive to their common activation stimuli, suggest that HCMV may induce anergy of CD4+ T cells. Anergy is

defined as a state of CD4+ T cells when they lose the ability to produce autocrine growth factors and proliferate in response to antigen stimulation[342]. Since CD4+ T cells play a central role in the functional immune response, our findings may partly explain the immunosuppression in HCMV infected patients.

When HCMV was treated with intravenous immunoglobulin (IVIG), anti-gB or anti-gH antibodies, the virus inhibitory effect was not affected. This indicates that viral glycoproteins such as gB and gH, which are important for virus attachment and entry into the host cells or main targets in the virus envelope to neutralizing antibodies are not involved in inhibition of CD4+ T cell proliferation.

As previously discussed, HCMV has been detected in various human malignancies, including GBM[114-116, 290-292, 294]. These growing evidences raise the possibility that chronic HCMV infection may contribute to carcinogenesis, perhaps in ways as other oncoviruses do. HCMV may also through indirect effects, such as on the immune system, contribute to tumor progression. Many of the biological responses caused by HCMV are directed towards the immune system of the host in order to avoid recognition and allow latency. Similarly, growing evidence suggest that despite the blood brain barrier, GBM tumors have multiple immunomodulatory abilities both locally at the tumor site and by affecting the whole immune system. In this study, we demonstrated that HCMV is able to block T cell proliferation and render T cells unresponsive. Earlier studies demonstrated that HCMV has multiple immunomodulatory abilities. For example, HCMV downregulates MHC class I and II complexes in the infected cells, hindering presentation of virus antigens to CD8+ and CD4+ T cells, and formation an organized adaptive immune response[253, 264]. It is interesting to note that microglia in GBM play an important role in antigen presentation, mainly though MHC molecules. Recent studies, suggest that microglia loose the capacity to express MHC molecules in GBM. This was suggested to be caused by increased production of immunosuppressive cytokines, such as TGF-β, IL-10 and PGE-2[343-345]. In addition, it has been shown that GBM tumor cells may skew the APCs towards a more immunosuppressive M2-like phenotype, hindering the normal antigen presentation in GBM tumors[346]. Additionally, glioma associated macrophages (TAMs) and microglia contribute to this immunosuppressive environment by producing immunosuppressive cytokines such as IL-10 and TGF-β [347, 348]. Likewise, HCMV would cause induced production of immunosuppressive cytokines, such as TGF-β and IL-10 by infected cells. The virus also produces an immunosuppressive IL-10 homologue (cmvIL-10) and a chemokine receptor homologue US28, which is believed to promote angiogenesis and is involved in carcinogenesis through COX-2 signaling[322]. In addition, HCMV produces an MHC class I homologue in order to avoid detection by NK cells[218]. It has been suggested and discussed earlier that HCMV also inhibits activation and cytotoxicity of NK cells[219]. Analogously, GBM cells inhibit NK cell cytotoxicity through overexpression of regeneration and tolerance factor (RTF) and downregulates MHC class I expression as well as show high surface expression of the nonclassic MHC class 1b molecule, which leads to inhibition of NK cell

proliferation [94, 95]. Additionally, GBMs are known to induce angiogenesis, which leads to tumor progression[12].

Intriguingly, there are many similarities in immunomodulatory and immunosuppressive abilities of HCMV and the immunphenotype that many GBM patients have. But what triggers what? The reported presence of HCMV proteins and nucleic acids in GBMs raise the possibility that GBM cells acquire certain immunosuppressive abilities from HCMV. Combination of immunomodulatory abilities of GBM and HCMV would have devastating consequences for the immune system of the host and may lead to tumor progression. Therefore, more knowledge is needed to understand whether GBM progression depends on the immunosuppressive phenotype that HCMV can induce in GBM patients, and to elucidate a potential role of this virus on cancer progression.

3.2 POOR SURVIVAL IN GLIOBLASTOMA PATIENTS IS ASSOCIATED WITH EARLY SIGNS OF IMMUNOSENESCENCE IN THE CD4 T-CELL COMPARTMENT AFTER SURGERY. (PAPER 2)

As discussed earlier, it is well known that GBM patients are immunosuppressed. GBM patients suffer from general T cell lymphopenia, possibly because of induced T cell apoptosis by monocytes[349], dysfunctional T cells, NK cells and monocytes, dysregulation of cytokine production and other immunosuppressive mechanisms [79, 350]. In essence, the immunological phenotype of GBM patients in many aspects is similar to that of a HCMV infected patients.

Since HCMV is frequently present in GBM and other tumors, we decided to investigate the immunological status of GBM patients with HCMV infected tumors. In paper 2, we analyzed the phenotype of T cells from GBM patients that participated in the VIGAS study. We then assessed certain immunological markers and their relevance to patient survival. The aim of the VIGAS study was to investigate the safety and efficacy of anti-viral treatment with Valganiclovir as an add-on to standard therapy in GBM patients with HCMV infected tumors. VIGAS was a randomized, double-blinded, placebo-controlled trial that recruited a total of 42 GBM patients with HCMV infected tumors to receive the anti-viral drug Valganciclovir or placebo, as add on to standard therapy. We collected blood and serum samples from GBM patients preoperatively, and at 3, 12 and 24 weeks after surgery to assess the immunological phenotype of the cells from these patients and correlate it to the overall patient survival.

We found clear signs of immunosenescence in the blood cells of GBM patients, as indicated by significantly higher levels of CD4+CD28- and CD4+CD57+ T cells. GBM patients with the immunosenescent phenotype had shorter survival time after diagnosis. We also observed that GBM patients had relative fewer CD3+ T cells compared to healthy controls. Although CD3+ T cells were reduced among GBM patients, the numbers of CD4+ and CD8+ T cells were not decreased as compared with controls, implying that CD3 depletion may reflect reduction of other cell types, such as NK cells[351, 352]. This result could also be affected by a loss of expression of the CD3 molecule on T cells. Earlier studies demonstrated that the loss

of CD3 expression might be associated with other virus infections, such as HIV[353, 354] and HTLV[355]. T cells undergo multiple processes during development and maturation. It has been suggested that during early development, thymocytes may lack CD3 expression, while expressing CD4 and CD8[356]. This raises a possibility that GBM patients may have a higher "turnover" of CD3+ T cells with faster elimination of these cells but slower maturation, shifting the balance to CD3+ T cell depletion. Another interpretation, although purely speculative, is that HCMV could affect CD3 expression on T cells in HCMV infected GBM patients in similar ways as in HIV and HTLV infections.

To assess the extent to which the immunological phenotype of GBM patients was correlated to patient survival, we analyzed T cell subsets at different times in long-term survivors (>20 months) and short-term survivors (<20 months). We found that GBM patients with short-term survival had lower levels of CD3+ T cells compared to patients with longer survival. We further observed a trend of a general increase in CD4+ T cells and a decrease in CD8+ T cells in GBM patients with shorter survival. This observation suggests that despite an increase in CD8+ T cells in the blood of GBM patients, these cells are probably dysfunctional or phenotypically altered and unable to contribute to a strong anti-tumor response. This may negatively affect patient survival.

Activation of CD8+ T cells is dependent on multiple consequent processes such as antigen presentation by APC on MHC class I molecules, recognition and signaling through TCR and engagement of the co-stimulatory molecule CD28, reinforced by cytokine stimulation[284]. As discussed earlier in this thesis, both HCMV infected cells and GBM cells exhibit reduced expression of MHC complexes. The loss of CD28 expression has been only observed on CD4+T cells from HCMV seropositive individuals[280], suggesting a viral influence on expression of co-stimulatory molecule CD28. Multiple immunosuppressive cytokines (for example IL-10, TGF-beta) are secreted both by HCMV infected cells and GBM cells with capacity to affect normal immunological responses. All these facts suggest a possible role of HCMV in development of an immunosuppressive phenotype in HCMV infected GBM patients, further allowing for tumor progression and poor patient outcome.

It has been described that T cell activation, proliferation and further differentiation into effector T cells depend on a functional CD28 co-stimulatory molecule[284]. Therefore, a loss of CD28 would have profound effects on normal T cell functions. It has been suggested that during persistent antigen stimulation, which leads to repetitive activation cycles of T cells, CD28 expression is progressively and irreversibly lost, leading to terminally differentiated T cells with shortened telomeres[357]. Therefore, the loss of expression of the CD28 molecule has been proposed as a hallmark of immunosenescence. In fact, more than 95% of T cells obtained from the blood of elderly individuals have lost CD28 expression. In contrast, more than 90% of T cells taken from young individuals expressed CD28 [358]. Interestingly, it has been demonstrated that CD28- T cells are absent in the umbilical cord and neonatal blood and gradually expand during life[284, 359]. Another study demonstrated that introduction of exogenous CD28 gene into human T lymphocytes in an *in vitro* model, delayed, but was

unable to permanently prevent cell senescence, indicating a central, but not the sole role of CD28 in replicative senescence[360].

We observed that the levels of CD4+CD28- T cells were increased at baseline and at 3 and 12 weeks after the surgery, with highest level at baseline. At 24 weeks after surgery we observed similar levels of CD4+CD28- cells compared with controls. After the surgical removal of more than 90% of GBM tumor mass, the expression of the CD28 molecule on T cells in the blood of GBM patients gradually returned to similar levels compared to healthy controls. This interesting observation suggests that HCMV-infected GBM tumors may have a direct immunomodulatory effect, which leads to the loss of the CD28 co-stimulatory molecule and contribute to immunosenescence. Removal of most of the HCMV-infected GBM tumor mass may have abrogated this immunomodulatory effect. To further investigate whether the loss of the CD28 molecule may be associated with patient survival, we analyzed CD4+CD28- and CD8+CD28- T cell subsets in blood at different times in long-term GBM survivors (>20 months) and short-term GBM survivors (<20 months). We observed significantly lower levels of CD4+CD28- in long-term survivors of GBM. We conclude that loss of CD28 expression on T cells is associated with poor outcome in GBM patients.

We also observed that the CD28 loss was positively correlated with CD57 expression in CD4 cells in the blood of GBM patients. Increased levels of CD57 expression together with CD28 loss in CD4+ T cells were also associated with shorter survival time of GBM patients. CD57 was first characterized as an NK cell marker, but later studies showed expression of CD57 in T cells and on cells of neural crest origin. The replicative history of T cells is defined by the increased expression of CD57 molecule and the loss of the CD28 molecule on the surface of T cells. [283, 361]. The expansion of CD28-CD57+ T cells are typical for HCMV seropositive individuals[242, 286]. Typical senescent features of CD57+ cells are limited proliferative capacity, shorter telomeres and decreased telomerase activity[284]. Thus, CD57+CD28- is a cell population associated with HCMV infection that may suggest a role of HCMV in the development of immunosenescence. We observed that the levels of CD4+CD57+CD28+ cells were increased in the blood of GBM patients at baseline and at 3,12 and 24 weeks after surgery. It is possible that chronic antigen stimulation by HCMV could contribute to the expansion of this cell population and further loss of CD28 co-stimulatory molecule.

Furthermore, we observed higher levels of γδT cells in the blood of GBM patients at baseline and at 3,12 and 24 weeks after surgery. Further investigation showed that this phenotype had no impact on GBM patient survival. γδT cells together with NK cells are considered to be the innate lymphocytes and are important components of an innate immune response. γδT cells use mechanisms that are MHC-independent and do not require processed antigens, recognizing for example stress-associated molecules and even NKG2D ligands[362]. It has been shown that a subset of γδT cells,Vδ2- cells target HCMV infected cells. HCMV infection may also induce a long-term expansion of circulating effector memory Vδ2- cells[250]. We observed higher levels of γδT cells in GBM patients without any impact on

patient survival, considering the presence HCMV infection in the GBM tumors, the virus may induce expansion of effector memory Vδ2-cells.

Another important subtype of immunological cells is the regulatory T cells (Tregs). Tregs cells play an important role in immunological tolerance and autoimmunity. They are known to have a general immunosuppressive capacity and therefore these cells may play a role in tumor-specific immune tolerance. In healthy individuals, Tregs maintain tolerance by exerting suppressive effects on effector T cells. In people with autoimmune diseases, Tregs fail to suppress autoreactive effector T cells, leading to damage of targeted cells or even cell death.[363]. The CD25 molecule and the transcription factor FoxP3 are considered to be well established markers of Tregs [364]. Unfortunately, the expression of FoxP3 was not investigated in the T cells of GBM patients enrolled in the VIGAS study and the conclusions about Tregs are therefore purely speculative. In our study, we have observed lower expression of CD25 molecule in the CD4 T cell compartment in the blood of GBM patients. This finding may suggest that the CD25+ subset of T regulatory cells is depleted in GBM patients. Thus, considering that this CD25+ T population may represent regulatory T cells, the immunophenotype of GBM patients resembles that of patients with autoimmune disease. In patients with autoimmune diseases, the Tregs fail to suppress autoreactive T cells because it has been suggested that in these patients Tregs have impaired function. With the knowledge of general immunosuppression of GBM patients, further impairment or depletion of Tregs in the context of HCMV-infected GBM tumors, may “unleash” the oncogenic abilities of both GBM and HCMV and lead to tumor progression.

GBM patients are treated with corticosteroids perioperatively and shortly after the surgery. This treatment is used in clinical settings to reduce the intracranial edema, caused by tumor expansion and surgery. It is well known that corticosteroid treatment may affect the immune system in various ways. Therefore, we examined whether corticosteroid treatment affected T cell phenotypes of GBM patients. The treatment showed no significant impact on T cell phenotypes during the study time.

The major side effects of the anti-viral drug Valganciclovir used in the study are kidney toxicity and pancytopenia, mostly affecting neutrophils and erythrocytes and thrombocytes. We further examined whether anti-viral treatment with Valganciclovir could also affect T cell phenotypes in GBM patients. This treatment had no significant impact on T cell phenotypes during the study.

Although a direct link between HCMV and immunosenescence remains controversial, HCMV affects the immune system in various ways. HCMV is the main driver of T-cell differentiation in the elderly, and possibly in patients with inflammatory diseases and HCMV-positive cancers. Thus, HCMV infection may lead to the typical immunological changes and the gradual immunosenescence seen in these patients. It is still unknown whether the immune phenotype associated with persistent HCMV infection in tumors affects the immune systems´ ability to combat the tumor, or through indirect effects affect the cellular biology of the cancer cells, permitting tumor progression, or whether HCMV itself directly

contributes to carcinogenesis. As HCMV proteins were detected in GBM tumors the main question remains: do GBM patients with chronic HCMV infection in their tumors have a poor prognosis due to the immune phenotype this virus may establish, or because the virus itself drives cancer progression?

3.3 CYTOMEGALOVIRUS INFECTION INDUCES A STEM CELL PHENOTYPE IN HUMAN PRIMARY GLIOBLASTOMA CELLS: PROGNOSTIC SIGNIFICANCE AND BIOLOGICAL IMPACT. (PAPER 3)

The role of HCMV in different types of cancers, including GBM is still debated mainly because different scientific groups have obtained different finding in this field. This discrepancy between the findings of different groups may be due to utilization of different non-optimized detection techniques for the virus. It has been shown that the vast majority of GBMs contain HCMV proteins and nucleic acids, suggesting the presence of HCMV in GBM[114-116]. An interesting recent finding from our group showed that the level of HCMV protein expression in GBMs is correlated to patient survival, implying a potential prognostic value of HCMV for patient survival in this fatal disease[118].

Despite the advances in the cancer field during the past decade, the survival of GBM patients remains at a dismal 14.6 months from the diagnosis[365]. GBM is well known for its phenotypic, morphologic and cellular heterogeneity, which contributes to constant therapy resistance[366]. Unfortunately, regardless treatment, recurrences are nearly unavoidable. GBM's aggressive nature and ability to survive oncological treatments, giving rise to tumor relapses, could be explained by the existence of CSC like cells within GBM (GSC)[365]. GSC are known to have pluripotent abilities: they proliferate and self-renew and they are able to differentiate and give rise to heterogeneous populations of cells that make up the bulk of solid tumors[99]. It has been shown that GSCs in GBMs in part resemble normal neural stem cells[100]. It has been demonstrated that HCMV interferes with development of neuroporgenitor cells by affecting the Notch pathway[367]. It is therefore pivotal to understand whether HCMV has a role in GSC formation in GBM, which would have an important clinical impact for GBM patients' treatment and survival.

Considering HCMV's oncomodulatory abilities in the context of GBM progression it is interesting to hypothesize that HCMV may play an important role in formation and maintenance of the GSC population. We therefore set out to investigate the ability of HCMV to induce a more aggressive CSC like phenotype in primary GBM cell lines. If so, this would provide a possible explanation for enhanced tumor progression and therapy resistance of HCMV infected GBM tumors.

In order to initially define the GSC population in GBM tumors we used the most abundant CSC marker, CD133. CD133 has been associated with normal neural stem cells and is expressed during embryonic development. Implantation of CD133 positive, but not CD133 negative cells in *in vivo* models gives rise to GBM tumors [96]. Although the exact function of CD133 in GBM remains poorly understood, silencing of CD133 expression impairs tumorigenic ability and self-renewal of GSC and decreases tumor formation *in vivo*[101].

We found that 90% of GBMs (19 out of 21 tumors) expressed CD133 and 95% (20 out of 21 tumors) expressed HCMV-IE proteins. As many as 80% of GBM tumors (16 out 20 tumors), co-expressed CD133 and HCMV-IE proteins. We also observed a positive correlation between expression of HCMV-IE and CD133 proteins, suggesting a possible functional relationship between these proteins. Theoretically, HCMV-IE proteins may induce expression of CD133 through their action as transcription factors able to regulate host gene expression[368]. Furthermore, both individual expression as well as co-expression of CD133 and HCMV-IE proteins were associated with shorter patient survival in GBM patients.

As discussed previously in this thesis, it has been debated which molecular markers are characteristic for GSC. It is believed that molecular markers associated with the maintenance of GSCs are differentially expressed in GSCs. These markers are categorized, according to their cellular localization: CD133 is a cell surface marker, Nestin is a cytoskeletal protein, SOX-2, OCT-4, Notch-1, Nanog are transcription factors and BMI-1 is a transcriptional suppressor [365]. While cell surface markers are important for detection and isolation of GSCs from GBM tumors, transcription factors are critical for maintenance the self-renewal, proliferation, survival and pluripotency of GSC[98]. In order to further investigate whether HCMV can induce GSC phenotype in GBM, we examined the expression of other CSC markers than CD133 after infecting primary GBM cell lines with HCMV. We observed that HCMV infection of primary GBM cell lines induced the expression of CSC markers, such as SOX-2, OCT-4, Notch 1, Nestin, BMI-1 and CD133. Earlier studies have demonstrated that GSCs have the ability to self-renew and form neurospheres when growing *in vitro*, and, when injected *in vivo,* give rise to heterogeneous tumors that resemble the original parent tumors[96]. We wished to further investigate whether HCMV could induce neurosphere formation in primary GBM cell lines. If so this would suggest that HCMV may be a possible contributing factor to an expansion of GSC subpopulation in GBM tumors and thereby contribute to the formation of an aggressive GBM phenotype poorly responding to chemotherapy. We observed that HCMV induced sphere formation in primary GBM cell lines when grown under nonadherent conditions, which as discussed earlier, is a typical behavior of GSC. These findings suggest that HCMV may induce or sustain an aggressive CSC like phenotype in GBM tumors and contribute to tumor progression.

The Notch pathway is known to be essential for CSC growth and maintenance of adult stem cells, such as interstitial[369] and neural stem cells[370]. Increased Notch activity has been demonstrated in a variety of tumors such as leukemia[371], breast cancer [372] and GBM[373]. Therefore, the Notch pathway is considered to play a crucial role in formation and maintenance of CSC and is suggested to contribute to therapy resistance in various cancer types, including GBM. Activation of Notch involves a few proteolytic cleavages that lead to release of the intracellular domains of Notch receptors and their nuclear translocation. This leads to a subsequent activation of Notch dependent transcription. The **γ** secretase is an enzyme that is needed for the last proteolytic cleavage of Notch, and this step is essential for Notch activation. **γ** –secretase inhibitors have been used to inhibit the Notch pathway in both *in vitro* and *in vivo* models [107]. In one animal study, inhibition of the Notch pathway

blocked proliferation of CD133 positive GSC cells and furthermore inhibited sphere formation as well as tumor growth in a GBM model, implying that the Notch pathway plays a central role in gliomagenesis[374]. Since we found that HCMV infection of primary GBM cell lines induced expression of Notch 1, we further investigated the role of the Notch pathway in GBM. We blocked Notch activation in primary GBM cell lines by treatment of cells with γ-secretase inhibitor. This treatment blocked sphere formation of GSC, indicating the importance of this pathway in proliferation of GSC.

In order to further investigate the possible role of HCMV in the formation of GSCs and to examine the effect of antiviral treatment on the development of GSC phenotype in GBM, we treated HCMV infected primary GBM cell lines with Ganciclovir (GCV). GCV is an inhibitor of the viral DNA polymerase and is used to treat HCMV infections in patients. Sphere formation ability was blocked by GCV treatment in superinfected primary GBM cell lines.

When primary GBM cell lines are obtained and established from the GBM tumors they express HCMV proteins but loose this expression during first days in culture (own unpublished observations). Primary GBM cell lines also lack the ability to induce sphere formation and further growth in neurospheres. But as the cells are grown in culture their phenotype may drastically change. Interestingly, we observed various expression levels of cytoplasmic HCMV-IE proteins without previous superinfection in primary GBM cell lines, especially at higher passage in culture. Additionally, we observed spontaneous sphere formation of primary GBM cell lines after multiple cell passages, approximately above passage 8 (unpublished data). These observations suggest a possible underlying endogenous " latent" HCMV infection, which is activated after a number of passages and phenotypical changes of the primary GBM cell lines in culture. This endogenous HCMV infection may further drive sphere formation in GBM cells and induce non-adherent growth as spheres.

In earlier studies, our group showed that HCMV can inhibit differentiation of neural precursor cells (NPC) into astrocytes and neurons *in vitro*. It has been suggested that late HCMV proteins played a major inhibitory role on cellular differentiation and HCMV also inhibited maturation and proliferation of NPC and induced apoptosis of these cells[375, 376]. These observations suggest that HCMV has an ability to block cellular differentiation and keep cells in an undifferentiated state. Therefore, we set out to investigate whether HCMV can affect the differentiation capacity of GSC into astrocytes and neurons. We found that the infected cells in the spheres remained undifferentiated, while cells not expressing HCMV-IE proteins were able to differentiate into neuronal and astocytic cells, as determined by assessment of neuronal and astrocyte markers. Our findings suggest that HCMV may be able to increase the GSC pool and maintain GSCs in an undifferentiated state.

Our results demonstrate that HCMV may play an important role in induction and maintenance of GSC subpopulation in GBM, possibly though activation of the Notch pathway. HCMV was able to keep GSC in an undifferentiated state, a characteristics which may contribute to the establishment of aggressive and therapy resistant cells in GBM tumors.

These observations imply that HCMV has an active role in GBM progression, rather than being an epiphenomenon of GBM. These findings have high clinical impact, suggesting a re-evaluation of current GBM therapies with consideration of anti-viral and Notch pathway-inhibiting drugs.

3.4 DISCORDANT HUMORAL AND CELLULAR IMMUNE RESPONSES TO CYTOMEGALOVIRUS IN GLIOBLASTOMA PATIENTS WHOSE TUMORS ARE POSITIVE FOR CMV. (PAPER 4)

Serology is a standard method still used in modern medicine for detection of previous or ongoing HCMV infection. Other methods for HCMV detection such as PCR for HCMV DNA and RNA help to optimize the detection of HCMV and confirm HCMV positivity in the patient. In this study, we set out to investigate the HCMV status in the blood of GBM patients, with HCMV-infected tumors, who were enrolled in the VIGAS study.

All 42 GBM patients enrolled in the VIGAS study showed HCMV protein expression in their tumors, as detected by IHC. Their blood cells were positive for HCMV DNA at least once during the study. However, in sharp contrast, only 71% of these GBM patients (30 out of 42) were HCMV IgG positive. Similar results were obtained using three different ELISA kits utilizing antigens of HCMV AD169 strain. However, when we used an in house ELISA kit with HCMV antigens from a clinical isolate for detection of IgG in sera from the 12 HCMV seronegative GBM patients, 42% of patients (5 out of 12) showed IgG positivity. Furthermore, 83% (10 out of 12) of these seronegative patients had T cells reactive against IE and pp65 HCMV proteins. Interestingly, 15% of HCMV RNA- positive patients lacked IgG against HCMV and would be considered to be HCMV seronegative. This discrepancy suggests that serology testing for HCMV is not a reliable method to use for detection of acute or previous HCMV infection. Existence of such discrepancy has been demonstrated earlier[377]. Viral reactivation was also earlier demonstrated in cells from an HCMV seronegative but HCMV DNA positive healthy blood donor[158]. The reason for this difference in the HCMV infection status measured by multiple methods is unknown. It may be explained by the existence of a unique viral strain, which is undetectable by standard serology tests or B cell tolerance with no or very low production of IgG. It can also be due to an unknown immune defect in GBM patients, possibly induced by their tumors or by a genetic phenotype pre-disposing for HCMV persistence. To further investigate the reason for this discrepancy, we examined T cell reactivity against HCMV proteins in the HCMV seronegative GBM patients. We observed that HCMV seronegative patients with positive HCMV DNA, RNA in blood and viral proteins in GBM tumors had T cells active against viral proteins, suggesting that HCMV exposed GBM patients may lack virus specific antibodies. It is fully possible that HCMV infected GBM patients have produced antibodies against HCMV earlier in their healthy life, but may have lost this ability during development of GBM in their brain. GBM patients also show signs of local immunosuppression at the tumor site[12]. Local GBM immunosuppression may permit active HCMV infection at the tumor site without functional antibody B cell response to HCMV in the blood. It is less possible that this discrepancy is due to low viral replication levels or protective immunity.

We also found additional evidence that HCMV is more active in GBM patients than controls. We observed higher IgM levels in 21% (9 out of 42) of GBM patients during the whole study time, compared in none of the healthy controls. Furthermore, HCMV RNA was detected in blood monocytes at least once during the study time in 63% of the patients; this is very rare among healthy blood donors. The higher IgM and RNA prevalence we observed in blood of GBM patients imply a higher activity of HCMV in these patients compared to controls.

In summary, we demonstrated a discrepancy between HCMV serology (detected by IgG and IgM) and detection of HCMV DNA and RNA as well as T cell activity against HCMV in blood of GBM patients. These findings suggest that serological detection of HCMV in GBM patients is an unreliable method. Furthermore, increased levels of HCMV IgM and HCMV RNA indicated an active HCMV infection in these patients and provide essential knowledge to further understand the immunological and viral phenotype of GBM patients, and will be important knowledge to improve current GBM therapies.

3.5 ENHANCED NEUTROPHIL ACTIVITY IS ASSOCIATED WITH SHORTER TIME TO TUMOR PROGRESSION IN GLIOBLASTOMA PATIENTS. (PAPER 5)

Different HCMV induced immunosuppressive mechanisms affecting different cell types are described earlier in this thesis, and may contribute to the general immunosuppression often observed in GBM patients. Despite persistent immunosuppression of GBM patients, it has been shown that these patients have increased levels of circulating neutrophils [378, 379]. Earlier studies demonstrated that increased levels of neutrophil infiltration in brain tumors were associated with higher grade of gliomas. GBMs are the most aggressive brain tumors, and they showed high levels of neutrophil infiltration[379]. HCMV is also able to affect neutrophils. As GBM tumors are frequently infected with HCMV, it is therefore of interest to understand what neutrophil and cytokine status HCMV-infected GBM patients have in their blood. The aim of this study was to investigate the activation status of peripheral neutrophils in blood and cytokine and chemokine patterns in plasma obtained from HCMV-infected GBM patients. We studied the aspects in blood obtained from GBM patients enrolled in the VIGAS trial.

We observed shorter overall survival and shorter time to tumor progression in patients with higher levels of neutrophil activity, as measured by increased expression of CD11b in neutrophils obtained from GBM patients. Remarkably, all patients with increased neutrophil activity had higher grade of HCMV infection in their GBM tumors, while just 69% of patents with low neutrophil activity had a high grade HCMV infection in their tumors (as detected by IHC). These results suggest that HCMV may contribute to increased neutrophil activity levels. HCMV has multiple immunomodulatory mechanisms, as discussed earlier in this thesis. As HCMV has an ability to induce neutrophil activation and inhibit their apoptosis, this may lead to more long-lived and over-reactive neutrophils that could contribute to tissue damage, inflammation, and allow for enhanced tumor progression[234]. In fact, we noted that 70 % (7 out of 10) of patients with GBM recurrence during the first 6 months after surgery,

presented with higher neutrophil activity at relapse. These observations indicate a possible correlation between increased neutrophil activity and GBM recurrence. Thus, increased neutrophil activity may be an indication of tumor progression in GBM patients. Patients with high neutrophil activity also had higher HCMV infection levels in their tumors, suggesting a possible role of the virus in GBM progression.

IL-12p70 is a neutrophil activator that is secreted mainly by DC and other APC in response to IFN γ stimulation by neutrophils and NK cells. This creates an autocrine loop, which again stimulates neutrophils to produce IL-12p70[380]. We therefore investigated the presence of this cytokine in the blood of GBM patients and detected increased levels of IL-12p70 in the patient group with enhanced neutrophil activity as well as in the whole GBM cohort. We observed that IL-12p70 levels were increased at 12 and 24 weeks after surgery in the patient group-receiving placebo, while the levels of IL-12p70 significantly decreased in the Valganciclovir treatment group at 24 weeks post surgery. Valganciclovir blocks the viral DNA polymerase and by inhibiting the viral activity, this treatment may decrease local inflammation at the tumor site. A decrease of IL-12p70 levels in the Valganciclovir treatment group versus the placebo group indicates a possible role of HCMV in neutrophil activation as well as in tumor progression. Earlier studies demonstrate that IL-12p70 secreted by neutrophils contributes to secretion of elastase by neutrophils in an autocrine manner, which may damage normal tissue and facilitate infiltration of GBM and tumor progression [378, 380]. We observed that patients with enhanced neutrophil activity and higher IL-12p70 levels had shorter time to tumor progression. IL-12p70 can also be released by neutrophils in response to infections. Earlier studies demonstrated IL-12 increases in response to Herpes simplex infection[381]. Consistent with this hypothesis, we observed lower levels of IL-12p70 in Valganciclovir treated patients compared to placebo-treated patients. Patients treated with the anti-viral drug Valganciclovir are expected to have lower HCMV activity and thereby less HCMV-induced inflammation in their GBM tumors.

We also found, elevated levels of the neutrophil attractant IL-8 and monocyte chemoattractant protein 1(MCP-1) in GBM patients compared to healthy controls, independent on neutrophil activity. MCP-1 may contribute to recruitment of T cells, monocytes, and dendritic cells to sites of infection as well as to enhance neutrophil and macrophage migration and infiltration. IL-8 is a chemokine that induces migration of neutrophils and other types granulocytes to the sites of infection. When investigating other cytokines and chemokines, we observed increased levels of IL-1b, IL-6, TNF-α, IFN-α and TGF-β in the whole GBM patient cohort compared to healthy controls. Levels of TGF-β, MCP-1, and IL-6 were significantly decreased during anti-tumor treatment in GBM patients. HCMV infection is known to affect the production of all of these cytokines[219, 228, 382]. Considering the known presence of HCMV in GBM tumors, these findings may suggest that the immune abnormalities in GBM patients with altered levels of inflammatory mediators may be affected by HCMV.

Summarizing our results, we found increased neutrophil activity in GBM patients in connection with tumor recurrence. These observations suggest that elevated neutrophil activity may be an early sign of tumor progression. GBM patients with shorter time to tumor progression had increased levels of IL-12p70 in their blood, a cytokine able to increase neutrophil activity. This would be consistent with high HCMV infection grade in their tumors. Indeed, high IL-12p70 levels were associated with increased neutrophil activity and correlated with poor patient outcome. Anti-viral treatment lowered levels of IL-12p70, which suggests that HCMV may have a role in neutrophil activation in GBM patients. Taken together our results suggest that neutrophil activation and HCMV infection may be involved in GBM progression, although more clinical and preclinical investigations are necessary to understand the mechanisms behind this association.

4 CONCLUSION

The work included in this thesis has been concentrated on increasing our understating of the potential role of HCMV in GBM. We characterized the immune phenotype of GBM patients and found striking signs of HCMV related immune phenotype characteristics, which were associated to tumor progression and poor patient outcome. HCMV also showed an ability to induce a more aggressive CSC-like phenotype in GBM that may contribute to therapy resistance. In the published scientific articles included in this thesis we have combined clinical research with cell molecular biology techniques to study the possible role of HCMV in GBM. Figure 8 is a schematic illustration summarizing the work of this thesis

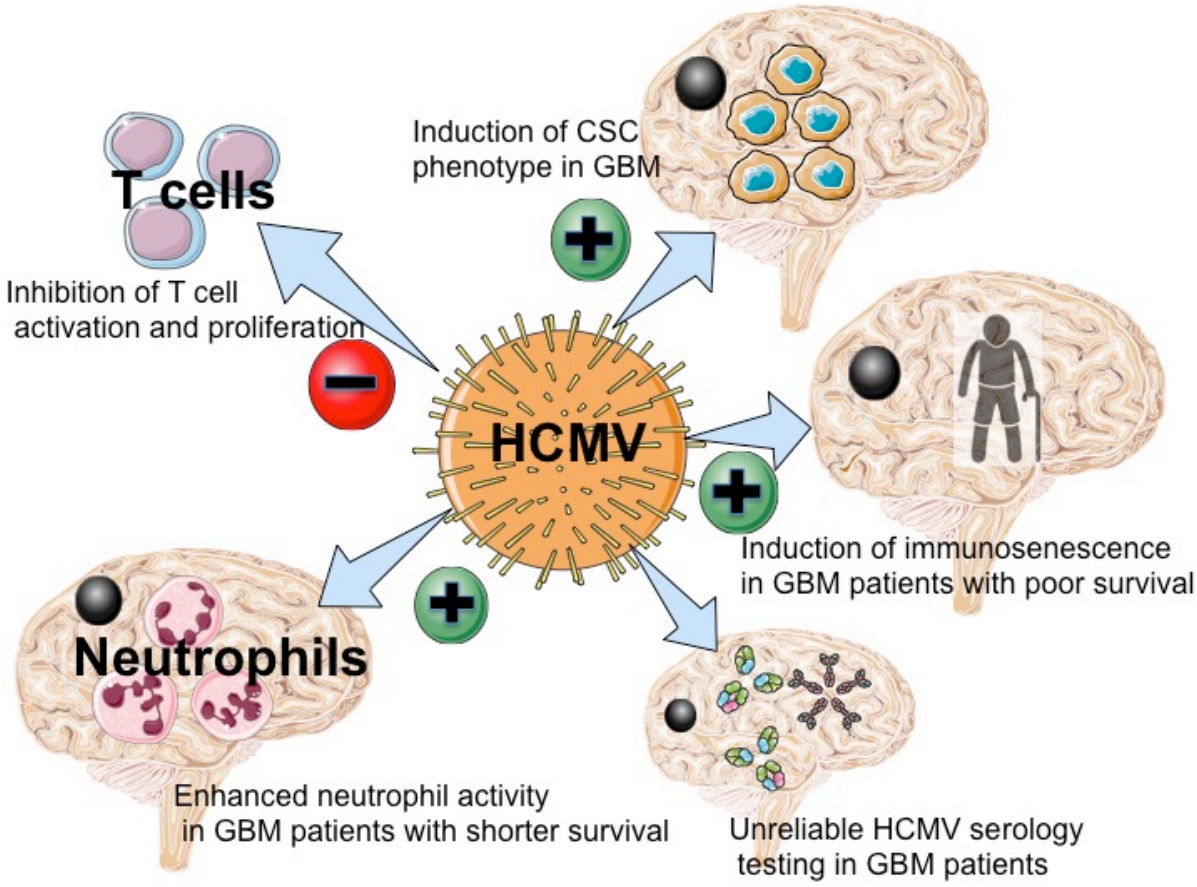


Figure 8. Summary of the work included in this thesis.

The major findings can be summarized as followed:

- HCMV inhibited proliferation of CD4+ T cells induced by phytohemagglutinin, concanavalin A, or phorbol myristate acetate. HCMV infected CD4+ T cells expressed the activation markers CD45RO and CD69, but produced reduced levels of cytokines and were anergic. This inhibitory effect was observed after HCMV infection while other viruses such as herpes simplex virus-1 and 2 or measles virus did not inhibit T cell prolferation. We conclude that HCMV has the ability to directly inhibit proliferation of CD4+ T cells and induce anergy of these cells. These results may partly explain the general immunosuppression often observed in HCMV-infected patients.

- We investigated the immune phenotype of 42 GBM patients diagnosed with HCMV-infected tumors. We found that HCMV positive GBM patients had lower levels of CD3+ T cells and increased levels of CD4+CD28-, CD4+CD57+ and CD4CD57+CD28+ T cells, which are signs of immunosenescene. Higher levels of CD4+CD28-, CD4+CD57+ and CD4CD57+CD28- T cells in the blood were also associated with shorter survival of GBM patients. We also observed higher levels γδT cells and lower levels of CD4+CD25+ cells. It is uclear whether immunosenescence is a pathological immune phenotype, especially arising in HCMV-infected GBM patients that may affect the ability of the host to combat the virus and the tumor, or if the changes in immunological phenotype are only an epiphenomenon of HCMV infection with no relevance in tumor progression. It is tempting to speculate that the reason why this immune phenotype arises is HCMV infection. Whether the immunological phenotype per se affects tumor progression or simply reflect the virus activity in the tumor will require further studies.

- We found that HCMV-IE proteins were co-expressed with the most abundant CSC marker in GBMs, CD133. Expression of HCMV-IE proteins and CD133 protein in GBMs were associated with poor patient survival. HCMV infection of primary GBM cell lines induced a CSC phenotype *in vitro,* by upregulating expression of CSC markers: CD133, SOX-2, Notch-1, OCT-4 and Nestin. HCMV infection of primary GBM cell lines induced neurosphere formation and expansion of spheres, which is a typical characteristics of GSC. Enhanced growth of spheres in HCMV infected primary GBM cell cultures was inhibited by a γ-secretase inhibitor, which targets the Notch pathway or the anti-viral drug Ganciclovir. HCMV infection inhibited differentiation of GSC into neurons and astrocytes and maintained GSC in an undifferentiated state. Our findings suggest that HCMV infection may induce a more aggressive CSC phenotype in GBM cells, which may lead to GBM tumor progression and enhanced therapy resistence.

- To asses the immune response to HCMV in HCMV infected GBM patients, we investigated viral serology status, HCMV DNA, RNA and HCMV specific T cell reactivity in blood cells of 42 patients with HCMV infected GBMs. We found, that despite the fact that all patients had at least one blood sample positive for HCMV DNA during the study, 29% of the patients were HCMV seronegative. 42% of the HCMV seronegative GBM patients were IgG positive, as determined through an ELISA test with antigens from clinical isolate. 83% of the HCMV seronegative patients had T cells in their blood that reacted against HCMV proteins (IE and pp65). Furthermore, 63% of the patients had HCMV RNA in their blood and 21% of the patients were IgM positive, compared to 0% of IgM positive individuals in the control

group. Our findings suggest that GBM patients have higher HCMV activity than healthy controls and that serology is an unreliable method for detection of previous or ongoing HCMV infection in GBM patients.

- We investigated the neutrophil activity and cytokine profile in the blood of patients with HCMV infected GBMs and correlated them to tumor progression. We found increased neutrophil activity, and increased levels of IL-12p70 in 12 out of 28 patients. GBM patients with increased neutrophil activity had high grade HCMV infection in their tumors and a shorter time to tumor progression. Anti-viral treatment with Valganciclovir possibly inhibited neutrophil activation because IL-12p70 levels were decreased in the Valganciclovir treated group, suggesting a potential role of HCMV in neutrophil activation. Our findings raise the possibility that neutrophil activation may be an early sign of GBM progression, and associated with more active HCMV infection in the tumor.

In summary, the work of my thesis has increased our understanding of the possible role of HCMV in GBM. The immune phenotype of GBM patients is consistent with a HCMV infection in these patients. We show that HCMV infection of GBM cells leads to a more aggressive phenotype with an enhanced pool of GSC. These findings may lead us towards the development of specific treatment protocols in GBM, combining anti-viral therapies with other conventional therapies to prolong the progression free survival of GBM patients, to enhance quality of life and maybe even in future cure GBM.

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